



Studies on microbial adhesion factors in smoking and Chronic Obstructive Pulmonary Disease (COPD)

by

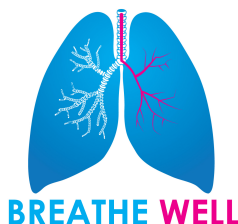
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Publications

***Data presented in these papers/abstracts are discussed in the thesis.**

Peer reviewed original research articles:

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***Shukla SD**, Muller HK, Latham R, Sohal SS, Walters EH. Platelet-activating factor receptor (PAFr) is upregulated in the epithelium of small airways and alveoli in smokers and COPD. *Respirology* 2016;21(3):540-510. DOI: 10.1111/resp.12709.

***Shukla SD**, Fairbairn RL, Gell DA, Latham R, Sohal SS, Walters EH and O'Toole RF. An antagonist of the Platelet-Activating Factor receptor inhibits adherence of both non-typeable *Haemophilus influenzae* and *Streptococcus pneumoniae* to cultured human bronchial epithelial cells exposed to cigarette smoke. *Int J Chron Obstruct Pulmon Dis*. 2016 July 11;1:1647-1655. DOI: <https://dx.doi.org/10.2147/COPD.S108698>

***Shukla SD**, Mahmood MQ, Weston S, Latham R, Muller HK, Sohal SS and Walters EH. The main rhinovirus respiratory tract adhesion site (ICAM-1) is upregulated in smokers and patients with chronic airflow limitation (CAL). (Submitted to *BMC Respiratory Research*).

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Shakti Dhar Shukla

Abstract

Background: Chronic obstructive pulmonary disease (COPD) is emerging as the third largest cause of human mortality worldwide, killing over more than 3 million people annually. It is defined as a chronic inflammatory lung disease characterized by airflow limitation that is not fully reversible. Tobacco smoking is the single most important causative factor for the development of COPD, especially in developed countries. Colonization/infection of the lower respiratory tract with microorganisms in COPD is likely to be one of the important factors driving inflammatory processes in the airways, possibly leading to long-term progression and exacerbations of the disease. Non-typeable *Haemophilus influenzae* (NTHi) and *Streptococcus pneumoniae* (*S. pneumoniae*) are the most important pulmonary bacteria during both stable phase of disease and exacerbations of COPD, but the underlying mechanisms for their prominence in these conditions remain elusive. Exposure to cigarette smoke has been shown to significantly increase pulmonary tissue bacterial infections (with both NTHi and *S. pneumoniae*) in cell culture and animal models, as well as human lung samples. Bacterial adherence to lung epithelium and subsequent tissue invasion is known to be a crucial step in the pathogenesis of respiratory infections. Notably, platelet activating factor receptor (PAFr) has been established as the primary attachment molecule for both NTHi and *S. pneumoniae*. Moreover, intercellular adhesion molecule-1 (ICAM-1) serves as an entry receptor for not only the majority (~60%) of human rhinoviruses (HRVs), but also for NTHi. Although the expression of these microbial receptors have been investigated in smokers, the expression pattern of these adhesion molecules in COPD has not been reported till date.

Aims: I have investigated the expression pattern of airway epithelial PAFr and ICAM-1 in both the large and small airways, as well as lung parenchyma in tissues from: smoker with normal spirometry, COPD subjects (smokers and ex-smokers), and a group with small dysfunction only. I have also investigated whether blocking PAFr reduces the bacterial adherence to bronchial cells pre-treated with cigarette smoke.

Methodology: I evaluated the expression of PAFr and ICAM-1 cross-sectionally in both large (bronchial biopsies, EBB and resected lung tissue, RT) and small airways (RT) from: 16 EBB/8 RT smokers with normal lung function (NLFS); 10 with smoking-related small airway narrowing only; 15 EBB/8 RT COPD smokers; 12 EBB/10 RT COPD ex-smokers, and compared these with 15 EBB/9 RT control tissues (NC). Anti-PAFr/ICAM-1 immunostaining on paraffin-embedded tissue was quantified using computer-aided image analysis. Moreover, human cultures bronchial epithelial BEAS-2B cells were exposed to cigarette smoke extract (CSE). PAFr and ICAM-1 expression levels were determined using immunocytochemistry and qPCR. The epithelial cells were challenged with either NTHi or *S. pneumoniae* labeled with FITC, and bacterial adhesion was measured using immunofluorescence. The effect of a well-evaluated antagonist of PAFr, WEB-2086, on binding of the bacterial pathogens to BEAS-2B cells was then assessed. With collaborators, I also carried out *in-silico* studies of the tertiary structure of PAFr and the binding pocket for PAF and its antagonist WEB-2086.

Results: Large airway epithelium showed markedly enhanced expression of PAFr in COPD-smokers especially ($p<0.005$) and also COPD-ex-smokers ($p<0.002$) compared to smokers with normal lung function. Moreover, significantly increased PAFr expression in small airway epithelium of all clinical groups was found compared with controls ($p<0.01$), but with no difference between smokers with or without COPD. Smoking history (pack-year) correlated significantly with PAFr expression in the currently smoking individuals, especially in NLFS ($r=0.9$; $p<0.002$). An increase above normal in PAFr-expressing cells in the airway sub-epithelial Rbm was only significant in COPD smokers ($p<0.007$). A lesser increase in PAFr-expressing cell in alveolar epithelium (compared to airway epithelium) was uniformly found in all clinical groups compared with normal controls ($p<0.01$). *In vitro* analyses showed that PAFr expression by bronchial epithelial cells was upregulated by CSE, and significantly associated with increased bacterial (both NTHi and *S. pneumoniae*) adhesion. WEB-2086, a known PAFr-antagonist, reduced the epithelial adhesion by both NTHi and *S pneumoniae* to levels observed for

non-CSE exposed control cells. Furthermore, WEB-2086 was non-toxic towards the bronchial epithelial cells. Moreover, *in silico* analyses allowed identification of the binding pocket for PAF/WEB-2086 in the predicted PAFr tertiary structure.

Significantly increased ICAM-1-positive cells were found in the epithelium of the both large ($p<0.006$) and small airways ($p<0.004$) of all clinical groups, compared to control tissue. Moreover, epithelial ICAM-1 expression was especially upregulated in the airway obstruction (CAL) group compared to smokers with normal lung function ($p<0.007$, for large airway expression; $p<0.02$, for small airway expression). Smoking history (pack-year) correlated significantly with ICAM-1 expression in both the large and small airway epithelium in the currently smoking individuals ($r=0.49$; $p<0.03$). In the sub-epithelial Rbm, increase in ICAM-1 expressing cells was only significant in small airways in the CAL group ($p<0.02$). The staining patterns were quite intriguing: in the CAL group especially, both basal and luminal areas of epithelial cells stained heavily for ICAM-1, but so did goblet cells and sub-mucosal glands. These features were little evident in either normal controls or NLFS subjects.

Conclusion: The expression of epithelial PAFr and ICAM-1 is upregulated in both the large and small airways in smokers, but markedly so in COPD. Increased expression of these microbial receptors in the respiratory tract could be crucial in viral and bacterial infections known to be important during the course of the disease.

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List of abbreviations

ACOS	Asthma COPD overlap syndrome
AECOPD	Acute exacerbations of COPD
AIHW	Australian Institute for Health and Welfare
AMs	Alveolar macrophages
ANOVA	Analysis of variance
ATS	American Thoracic Society
ATT	α 1-antitrypsin deficiency
BAL	Bronchoalveolar Lavage
BEGM	Bronchial epithelial cell growth medium
BOLD	Burden of Obstructive Lung Disease
BSA	Bovine Serum Albumin
BTS	British Thoracic Society
CAL	Chronic airflow limitation
CAP	Community acquired pneumonia
CDHR3	Cadherin-related family member 3
ChoP	Phosphorylcholine
CFU	Colony forming unit
COPD	Chronic Obstructive Pulmonary Disease
CSE	Cigarette smoke extract
DAMPs	Damage associated molecular patterns
DALYs	Disability adjusted life years
DLco	Diffusing capacity of carbon monoxide
DNA	Deoxyribonucleic Acid
DAB	Diaminobenzidine
EBB	Endobronchial biopsy
ERS	European Respiratory Society
FEV1	Forced expiratory volume in one second
FEF₂₅₋₇₅	Force mid-expiratory flow rate
FER	Forced expiratory ratio
FP	Fluticasone propionate
FVC	Forced Vital Capacity
EMT	Epithelial-Mesenchymal Transition
ECM	Extracellular Matrix
FITC	Fluorescein isothiocyanate

GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GOLD	Global Initiative for Chronic Obstructive Pulmonary Lung Disease
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GPCR	G-protein coupled receptor
H₂O₂	Hydrogen peroxide
H & E	Hematoxylin and Eosin
HBD-2	Human β -defensin-2
HRP	Horseradish Peroxidase
HRV	Human Rhinovirus
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
ICS	Inhaled Corticosteroids
IL	Interleukins
IFN-γ	Interferon γ
Ig	Immunoglobulin
LABA	Long Acting Beta Agonist
LAMA	Long Acting Muscarinic Antagonist
LDLR	Low-density lipoprotein receptor
LFA-1	Lymphocyte function-associated antigen 1
LLN	Lower limit of normal
LRTI	Lower respiratory tract infections
LTB₄	Leukotriene B ₄
LPS	Lipopolysaccharide
MCP	Monocyte chemotactic protein
MHC	Major-histocompatibility-complex
MIP	Macrophage inflammatory protein
MMPs	Matrix metalloproteinases
MPO	Myeloperoxidase
mRNA	Messenger Ribonucleic Acid
NC	Normal control
NCDs	Non-communicable diseases
NE	Neutrophil elastase
NF-κB	Nuclear factor kappa B
NLFS	Normal lung function smoker
NLRs	Nod-like receptors
NO	Nitric Oxide

NTHi	Non-typeable <i>Haemophilus influenzae</i>
O₂-	Superoxide Anions
OR	Odds ratio
PAMPs	Pathogen-associated molecular patterns
PAF	Platelet activating factor
PAFr	Platelet activating factor receptor
PEF	Peak expiratory flow
pIgR	Polymeric Immunoglobulin receptor
PM	Particulate matter
PRRs	Pattern recognition receptors
QoL	Quality of life
qRT-PCR	Quantitative Reverse-Transcriptase–Polymerase-Chain-Reaction
RANTES	Regulated on activation, normal T cell expressed and secreted
Rbm	Reticular Basement Membrane
RLRs	RIG-like receptors
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
RR	Relative risk
RSV	Respiratory Syncytial Virus
RT	Resected tissue
SAD	Small airway disease
SE	Standard error
SES	Socioeconomic status
SHS	Second hand smoke
TAHS	Tasmanian Longitudinal Health Study
TB	Tuberculosis
TGF-β1	Transforming growth factor-Beta1
TLRs	Toll like receptors
TNF-α	Tumour Necrosis Factor-Alpha
URTI	Upper respiratory tract infections
WHO	World Health Organization

Chapter 1

COPD: An introduction

1.1 Characteristics of COPD

1.1.1 Clinical

In the current perspective, the widely used functional definition of COPD drafted by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) defines COPD as "a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases" (GOLD 2015). Although the definition includes 'inflammatory' response as a hallmark of COPD, the data on inflammatory cellular profile in the airway wall is contradictory. Based on our laboratory findings (Figure 1.1), we conclude that the classic inflammation may not play a central role in COPD, at least in the early stages of the disease and in the airway wall (Sohal et al. 2015).

Common signs and symptoms of COPD include chronic cough, shortness of breath and mucus hypersecretion. The major pathologic changes of COPD are observed in the airways. Lung parenchyma and pulmonary vasculature could also show significant pathological manifestations.

The tracheobronchial tree is a highly branched system of conducting tubes, which can be divided schematically into large airways (airways with cartilage in their walls, usually >2mm in internal diameter) and small airways or bronchioles (no cartilage in their walls, usually <2mm in internal diameter) (Bohadana, Teculescu & Martinet 2004). The landmark study by Hogg et al. in late 1960s suggested that earliest pulmonary changes and airflow obstruction in smoking-related COPD occurs mainly at the level of the small conducting airways and surrounding alveoli, which become the major site of airflow obstruction (Hogg, Macklem & Thurlbeck 1968). Briefly, various cellular and biochemical processes in the lung result in progressive thickening of the wall in small airway with luminal narrowing, fibrosis and small airway obliteration

(~50%) (Belperio et al. 2003; Hogg et al. 2004; Hogg, McDonough & Suzuki 2013). These changes increase airway resistance and airflow obstruction. Previous experimental measurements suggest that as high as 30-40% of airway resistance could be attributed to small airways (Takishima, Yanai & Sasaki 1991), which is considerably higher than what was estimated earlier (~20%) (Hogg, Macklem & Thurlbeck 1968).

Airway inflammation has become something of a dogma in COPD literature but is really a phenomenon of the airway lumen until the disease is very severe (Hogg et al. 2004). More marked in the airway wall is remodeling in the form of epithelial goblet cell hyperplasia (bronchitis), epithelial squamous metaplasia and submucosal gland hyperplasia in majority of patients, emphysema develops only in a subset of individuals, possibly as secondary phenomena.

The dilatation and destruction of the gas-exchanging tissue beyond the terminal bronchioles is termed “emphysema” (McDonough et al. 2011). Emphysema is defined in pathologic terms as “alveolar wall destruction with irreversible enlargement of the air spaces distal to the terminal bronchioles and without evidence of fibrosis” (Snider et al. 1985), and in general, occurs a decade later than airway narrowing (Dunnill 1969). Morphologically, there are two main subtypes of emphysema (Kim, WD et al. 1991). The centrilobular (or centriacinar) emphysema is most closely associated with cigarette smoking, causing more severe small airways obstruction. The panlobular (or panacinar) emphysema is associated with α 1-antitrypsin deficiency, resulting in an even dilatation and destruction of the entire acinus (Litmanovich, Boisselle & Bankier 2009).

Changes in the pulmonary vasculature include intimal hyperplasia and smooth muscle hypertrophy/hyperplasia, likely attributed to chronic hypoxic vasoconstriction of the small pulmonary arteries (Harkness et al. 2014). Emphysema can lead to loss of the associated areas of the pulmonary capillary bed and pruning of the distal vasculature, which can be detected radiographically.

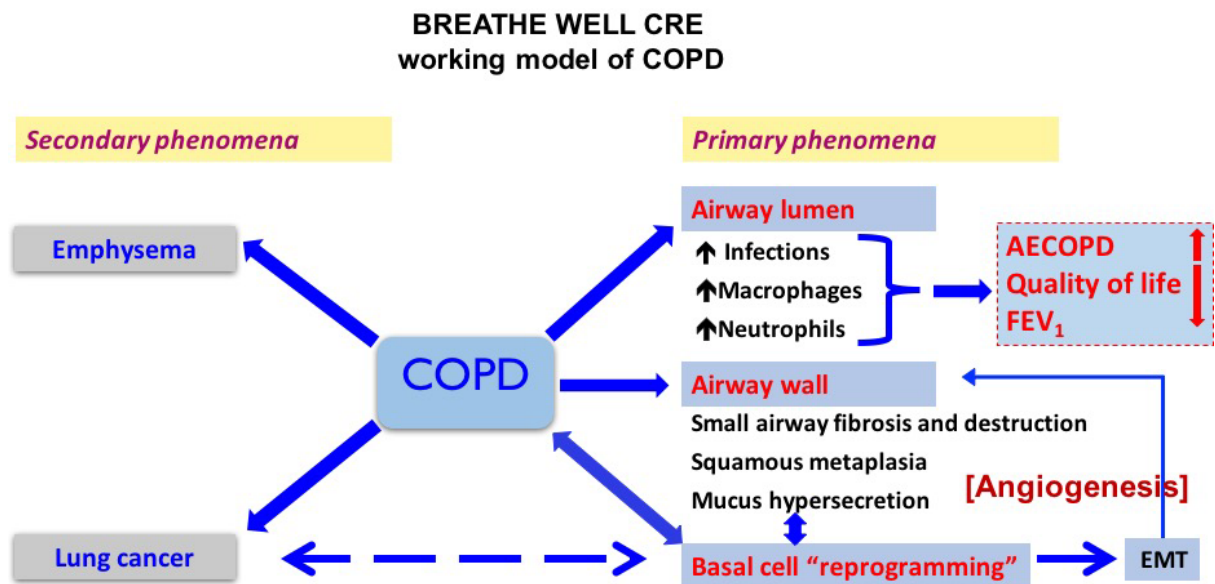


Figure 1.1. Clinical characteristics of COPD.

Primary phenomena include fibrosis and obliteration of small airways, as well as other airway remodeling changes such as squamous metaplasia, mucus hypersecretion and goblet cell hyperplasia in large airways. Airway luminal inflammation becomes prominent as the disease progresses, with inflammatory cells probably related to chronic infections with ‘respiratory pathogens’. These changes are associated increased risk of acute exacerbations of COPD, decreased quality of life and accelerated decline of lung function. Basal cells in the airway epithelium are increasingly regarded as extremely important in airway remodeling. One manifestation of this is the change on the features of the epithelium, but also include epithelial-mesenchymal transition (EMT), type II or III, categorized on the basis of presence (type III) or absence (type II) of angiogenesis.

Secondary phenomena include emphysema (enlargement and destruction of terminal bronchioles and alveoli), and lung cancer, potentially related to EMT, especially type III with angiogenesis in the reticular basement membrane (Rbm) and epithelium (Mahmood et al. 2015).

1.1.2 Physiological (Spirometric classification)

By standard definition, diagnosis of COPD requires the presence of poorly-reversible airflow obstruction using an objective spirometry assessment (Global Strategy for the Diagnosis 2015). The introduction of an obstructive index, i.e., the ratio of forced expiratory volume in 1 second (FEV_1) to the forced expiratory vital capacity (FVC) (Hyatt & Black 1973), and its more modern equivalent of scalloping out of the expiratory limb of the maximal flow-volume curve (Johns, Walters & Walters 2014) have become pivotal diagnostic indices in clinical medicine. Forced expiratory volume in 1 second (FEV_1) indicates the volume of air exhaled under forced conditions in the first second. Forced vital capacity (FVC) is the determination of the vital capacity from a maximally forced expiratory effort. Both are measured after a full inspiration. Spirometry is a safe, non-invasive, practical and reproducible breathing test that can be performed quickly. Modern spirometers have the added advantages of yielding immediate computer-generated feedback to the operator on the quality and repeatability of the test, as well as real-time graphical display of the flow-volume curve. Spirometry is performed both before and after bronchodilator administration to confirm whether airflow limitation is present and whether it is partially-, fully- or non-reversible. The ratio of these two measures, i.e., FEV_1/FVC is termed the forced expiratory ratio (FER), and a FER of <0.7 confirms the presence of airway obstruction, while $FEV_1\%$ predicted is used to classify COPD severity (mild $\geq 80\%$, moderate 50-80%, severe 30-49%, very severe $<30\%$).

The social and economic burden of COPD occurs predominantly in Stages II and higher. However, the confirmation of COPD based on specific post-bronchodilator FER of <0.7 has certain limitations. The fixed ratio excludes normal age-related changes in airflow limitation (Ito & Barnes 2009). Evidently, there is a decline in FER associated with normal ageing. In healthy non-smoking adults, the decrease in FEV_1 is about 30 mL/year with an upper limit of about 50 mL/year (Kerstjens et al. 1997). As a consequence, even in normal people, the lower limit of normal for FER also decreases with age and should be accounted for to confirm COPD (Hankinson, Odencrantz & Fedan

1999). Therefore, establishing COPD based solely on FER is likely to misclassify some healthy elderly as diseased (Stanojevic et al. 2008; Vaz Fragoso et al. 2010). More appropriate classification rules for COPD classification based on a lower limit of normal (LLN) i.e., more than $1.64 \cdot SD$ below the predicted level (5th percentile for age) has been proposed (Schermer & Quanjer 2007). This has not been widely accepted and many clinicians argue that it is important to accept that accelerated ageing of the airways/lungs even in non-smokers is a significant issue clinically, and if symptomatic, should be indeed treated as COPD.

Detection of small airways function by spirometry is by measuring the forced expiratory flow over the middle half of the FVC ($FEF_{25-75\%}$) and forced expiratory flow at 75% of the FVC ($FEF_{75\%}$) (McFadden & Linden 1972). However, these measures do not contribute to clinical decision-making due to the dependency on the measurement of FVC and lack of repeatability (Quanjer et al. 2014). This area is currently being reconsidered with better indices of spirometric small airway function being proposed (Johns, Walters & Walters 2014). In addition, newer technology has been proposed (Milne & King 2014). It is worth emphasizing that major pathological damage has to take place in small airways well before dynamic lung function indices become abnormal. With current measurements, at this stage, COPD often advances to a moderate stage (Hogg, McDonough & Suzuki 2013) before detection (Johns, Walters & Walters 2014).

Table 1. Staging of disease severity.

Disease Severity	(FEV ₁ Predicted)			
	ATS	ERS	BTS	GOLD
Stage 0: at risk	—	—	—	Normal spirometry; Chronic symptoms (cough, sputum production)
Stage I: mild	≥50%	≥70%	>80%	≥80% with or without chronic symptoms
Stage II: moderate	35%-49%	50%-69%	31%-80%	50%-79% with or without chronic symptoms
Stage III: severe	<35%	<50%	≤30	30%-49% with or without chronic symptoms
Stage IV: very severe	Terminally ill			<30%

FEV₁- forced expiratory volume in 1 second; ATS- American Thoracic Society; ERS- European Respiratory Society; BTS- British Thoracic Society; GOLD- Global Initiative for Chronic Obstructive Lung Disease.

Despite the availability of all these measures, COPD still remains substantially under-diagnosed in the primary care settings, particularly due to underuse of spirometry and poor interpretation of spirometric data (Walters, JA et al. 2008). Recently, Johns et al. summarized the data on use of spirometry in current perspective and stressed on the importance of early diagnosis of COPD based on quantification of the degree of concavity in the spirometric expiratory flow-volume curve (Figure 1.2) (Johns, Walters & Walters 2014).

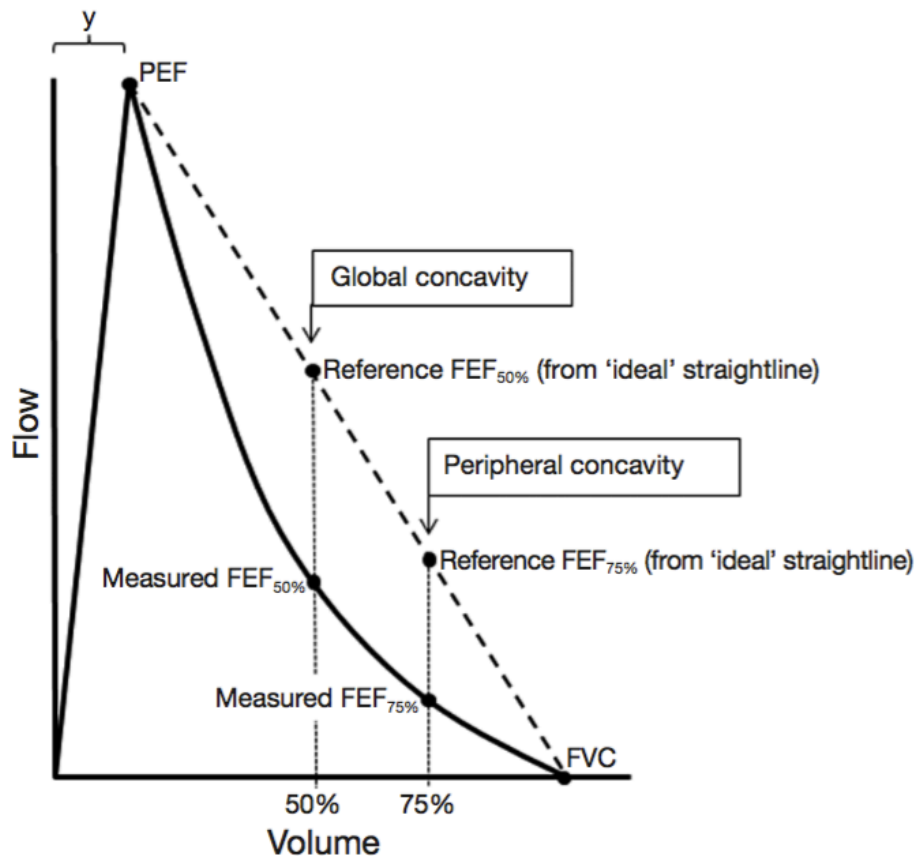


Figure 1.2. Variables used to quantify global and peripheral concavity.

A novel way of interpreting the expiratory limb of the F-V curve. Measured $FEF_{50\%}$ and measured $FEF_{75\%}$ are the forced expired flows when 50% and 75% of the FVC has been expired. Reference $FEF_{50\%}$ and Reference $FEF_{75\%}$ are the reference flows that would be obtained if the flow-volume curve had zero curvature i.e., a linear descending limb (dotted line). The variable, y , is the volume to peak expiratory flow (PEF); a value of 0.6 L can be assumed for this. In this example, global concavity is approximately 50 Units and peripheral concavity is approximately 65 Units. Such an analysis may markedly improve the pick-up of early COPD (by perhaps 25%).

From Johns, Walters and Walters (2014) with permission from AME Publishing Company.

In most specialist practice, a further assessment of lung physiology in COPD also takes into account lung hyper-inflation (emphysema or just air-trapping due to small airway disease) using a body-plethysmograph, and also the lung diffusion capacity (oxygen-absorbing capacity) as a sensitive measure of

extent of emphysema. The diffusing capacity for carbon monoxide (DL_{CO}) is an index of the degree of anatomic emphysema in smokers with airflow limitation, but is not needed for routine assessment of COPD. The DL_{CO} decreases in proportion to the severity of emphysema; however, it can be difficult to detect mild emphysema because it is neither a sensitive nor a specific test (Bailey 2012).

1.2 Epidemiology of COPD

1.2.1 Global burden of COPD

The highly cited global burden of disease study (GBD) highlights COPD as being the sixth leading cause of death in 1990, increasing to fourth since 2000, and projected to become the third by 2020 (Lopez et al. 2006). The Burden of Obstructive Lung Disease (BOLD) initiative was designed to be somewhat more accurate and develop standardized methods for estimating COPD prevalence world-wide that would be practical for use in countries with differing economic development profiles and also to then estimate the economic burden of COPD (Buist et al. 2005). BOLD study data from 12 sites ($n=9425$; in China, Turkey, South Africa, Austria, Iceland, Germany, Poland, Norway, Canada, USA, Philippines and Australia), up to 2006, reported the estimated population prevalence of COPD GOLD Stage II and higher as $10.1\pm 4.8\%$ (SE) overall ($11.8\pm 7.9\%$ for men and $8.5\pm 5.8\%$ for women). The prevalence of COPD increased with age and pack-years of smoking, although other risk factors, such as biomass use for heating and cooking, chronic occupational exposure to dusty environments and tuberculosis, could also contribute to location-specific variations in disease prevalence (Buist et al. 2007).

Halbert and colleagues (Halbert et al. 2006) conducted a systematic review and random effects meta-analysis quantified the global prevalence of COPD (62 studies published during 1990-2004 from 28 different countries). The prevalence of physiologically defined COPD (GOLD criteria) in adults (≥ 40 years) was again approximately 9–10%. The authors also report the

prevalence of chronic bronchitis alone (6.4%, 38 studies) and emphysema alone (1.8%, 8 studies).

Rycroft et al. reviewed 133 studies published between 2000-2010 the trends in the COPD prevalence (80 articles), incidence (15 articles), and mortality (58 articles) in Australia, Canada, France, Germany, Italy, Japan, The Netherlands, Spain, Sweden, the United Kingdom, and the USA (Rycroft et al. 2012). COPD prevalences ranged from 0.2% (Japan) to 37% (USA), but varied widely across countries and populations reported, COPD diagnosis and classification methods and age groups reported. The burden of COPD was more commonly reported in older populations (>75 years). Moreover, the prevalence of COPD has increased over time, although the rate of increase has declined in recent years, particularly among men.

Similar to prevalence, the overall mortality rate varied between countries, ranging from 3–9 deaths per 100,000 in Japan to 7–111 deaths per 100,000 in the USA (Rycroft et al. 2012). Respiratory diseases, including asthma and COPD, were the third leading non-communicable diseases (NCDs) causing 4.2 million deaths globally in 2008 and are expected to become more severe in near future (WHO 2014b). Globally, COPD affects approximately 329 million people, which accounts for nearly 5 percent of the total population (Vos et al. 2012). In terms of mortality, COPD resulted in 2.9 million deaths in 2013 alone, which increased from 2.4 million deaths in 1990, which was published as an international collaborative global burden of disease project ('Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013' 2015).

In addition to mortality and morbidity burden of COPD, understanding the economic implications associated with the disease and its treatments is as important as understanding their clinical impact. Medical expenditures for treating COPD (chronic medication needs, frequent exacerbations, hospitalization and ambulatory oxygen therapy required by those with advanced COPD) and the indirect opportunity-lost costs of morbidity can

represent a substantial economic and social burden (Ramsey & Sullivan 2003). In terms of global expenditure on COPD, the cost is expected to rise from US\$2.1 trillion in 2010 to US\$4.8 trillion in 2030, half of which is expected to arise in developing countries (Lomborg 2013). Of the US\$2.1 trillion expenditure in 2010, approximately US\$1.9 trillion accounted for direct costs such as medical care, while US\$0.2 trillion was spent as indirect productivity losses, such as absence from work (Bloom 2011).

1.2.2 National burden of COPD in Australia

In Australia, the death rates due to COPD have approximately halved between 1979 and 2011 among males, although it increased between 1979 and 1997 among females before starting to decline (Figure 1.3). Moreover, deaths due to COPD are higher among people of indigenous origin compared to non-indigenous Australians (AIHW 2012). All of this reflects current and legacy of smoking rates.

Despite falling death rates, COPD is still a leading cause of death and disease burden, after heart disease, stroke and cancer. The principal risk factor for COPD in Australia is indeed cigarette smoking, like other developed and probably under-developed countries. Chronic respiratory diseases (including COPD) are the fourth leading cause of deaths annually (approximately 6% of total deaths in 2008) (AIHW 2012). COPD alone is the fifth leading cause of mortality, causing 6,122 deaths in 2011 (AIHW 2012). However, the median age of death from COPD was reported to be 81 years in 2007–2011. Moreover, data from The Sax Institute's 45 and Up Study (n= 204,953; aged ≥45 years) has found not only that two-thirds of deaths in current smokers can be directly attributed to smoking, which is much higher than the earlier international estimates of 50%, but in addition, current smokers are estimated to die an average of 10 years earlier than non-smokers (Banks et al. 2015). The age of 45 seems to be something of a watershed: if a smoker stops smoking, then they are largely unscathed, but beyond that real trouble starts.

Australian BOLD study estimates that approximately 14.5% (one in seven) Australians >40 years have airflow limitation of their lungs, which increases to 29.2% in Australians >75 years. Moreover, around 7.5% Australians (>40 years) have subjective symptoms and it is estimated that about half of them are under-diagnosed (Toelle et al. 2013).

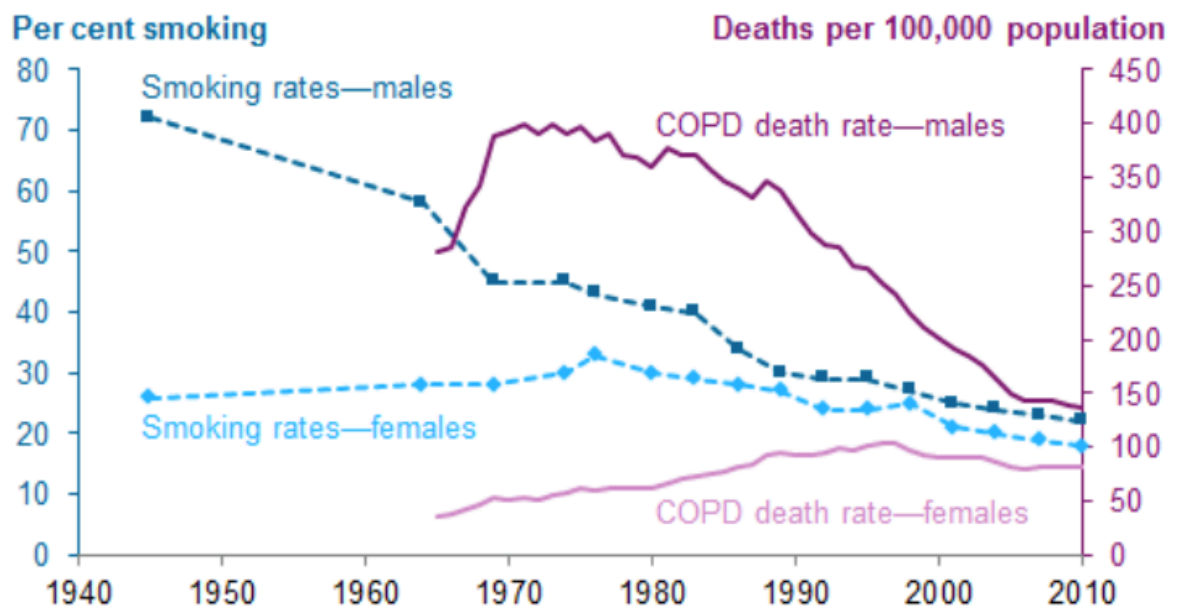


Figure 1.3. COPD death rates in Australia.

The data is presented on a 3-year moving average, and smoking rates, 1945 to 2010. The two are obviously closely linked, albeit with about 20 years lag-phase. *Source: (AIHW 2012).*

In terms of total costs, COPD is more costly per case than cardiovascular disease (CVD), osteoporosis or arthritis and therefore exerts a huge economic burden on Australia. COPD is Australia's second leading cause of avoidable hospital admission (Glover et al. 2007). In 2008 alone, the total economic impact of COPD was estimated to be AU\$98.2 billion. AU\$8.8 billion was attributed to direct financial costs and AU\$89.4 billion to the loss of wellbeing and productivity. Annually, the direct cost to the Australian health care system is estimated to be \$900 million with hospital use contributing the largest share of health spending (\$473 million) ('Access Economics' 2008).

1.3 Risk Factors for COPD

The risk factors for COPD can be broadly categorized as exposures and host-related factors (Figure 1.4). Various strategic steps could be employed to minimize the exposures, at least partially, for each exposure category. However, host-related (genetic) factors possibly leading to lung pathology and appearance of symptoms are extremely difficult to control. These factors are briefly summarized here, with a major focus on cigarette smoke exposure.

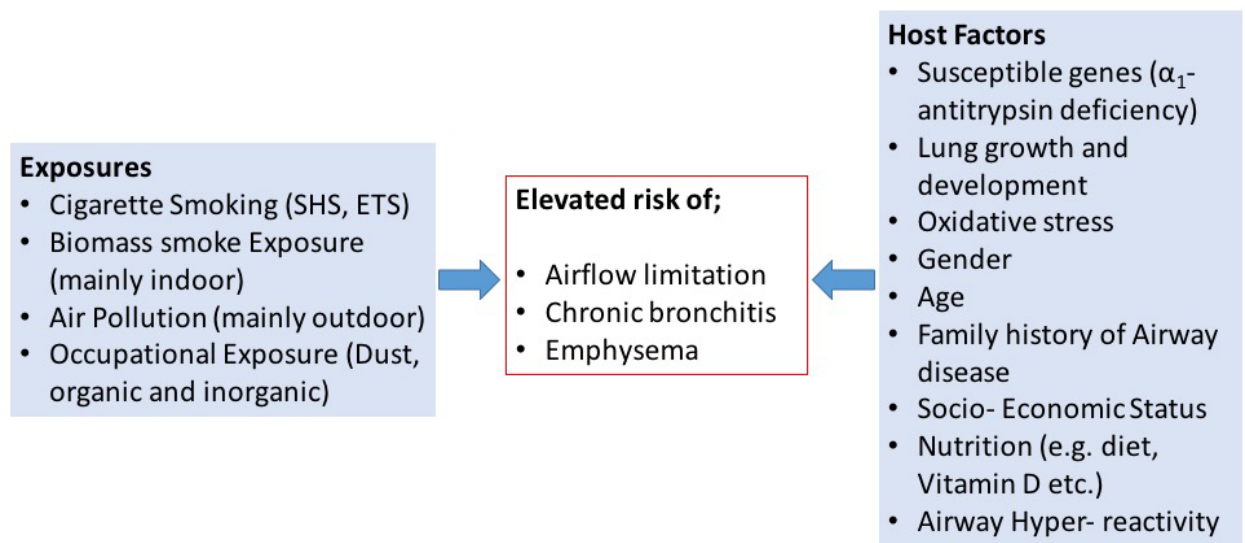


Figure 1.4. Known risk factors for COPD.

1.3.1 Exposure

As mentioned earlier, under ideal conditions, pulmonary function increases during childhood and adolescence (FEV_1), up to the age of 18–22 years and then plateaus before starting to decline as a normal process of ageing (Eisner et al. 2010), probably after about age 30–35. Various exposures that affect the normal lung growth phase leading to decreased maximal FEV_1 , shorten the plateau period, or increase the subsequent decline in pulmonary function could lead to COPD. In this section, the role of various exposures will be reviewed briefly.

1.3.1.1 Cigarette Smoke (Active and passive smoking)

Exposure to cigarette smoking (direct or indirect) is the principal causal factor for COPD. There are approximately 1.1 billion daily smokers globally, which could increase to 1.6 billion daily smokers by 2025 (Bilano et al. 2015). Currently, WHO estimates about 6 million annual deaths due to tobacco use (both smoking and smokeless). This total also includes about 600,000 people, estimated to die from the exposure of second-hand smoke. Mathers and Loncar (Mathers & Loncar 2006) project total tobacco-attributable deaths to rise from 5.4 million in 2005 to 6.4 million in 2015, which will further increase to 8.3 million in 2030 (7.4 million to 9.7 million). Tobacco-attributable deaths are projected to decline by 9% between 2002 and 2030 in high-income countries, but to double from 3.4 million to 6.8 million in low- and middle-income countries. This is attributed to increasing total and smoking populations, lack of awareness and ineffective tobacco control policies in developing countries. In a systematic review with meta-analysis of the epidemiological evidence relating smoking to COPD, chronic bronchitis (CB; chronic cough and sputum production) and emphysema, Forey et al. analyzed 218 studies (133 for COPD, 101 for CB and 28 for emphysema) (Forey, Thornton & Lee 2011). The authors reported that relative risk estimates are elevated for **ever smoking** (RR for COPD: 2.89; 95%CI: 2.63-3.17, n=129; RR for CB: 2.69; 95%CI: 2.50-2.90, n=114; RR for emphysema: 4.51; 95%CI: 3.38-6.02, n=28), **current smoking** (COPD: 3.51; 95%CI: 3.08-3.99; CB: 3.41; 95%CI: 3.13-3.72; emphysema: 4.87; 95%CI: 2.83-8.41) and **ex-smoking** (COPD: 2.35; 95%CI: 2.11-2.63; CB: 1.63; 95%CI: 1.50-1.78; emphysema: 3.52; 95%CI: 2.51-4.94).

Ng et. al. estimated the prevalence of daily smokers by age and sex and the number of cigarettes/smoker/day for 187 countries from 1980 to 2012. The authors show that the number of daily smokers (>15 years) increased significantly, from 721 million (95% uncertainty interval [UI], 700 million–742 million) in 1980 to 967 million (95%UI, 944 million–989 million) in 2012 (Ng et al. 2014). Although the numbers are predicted to increase, due to population growth, the prevalence of daily tobacco smoking in the same population decreased from 41.2% (95%UI: 40.0%-42.6%) in 1980 to 31.1% (95%UI: 30.2%-32.0%) in 2012 for men and from 10.6% (95%UI: 10.2%-11.1%) to

6.2% (95%UI: 6.0%-6.4%) for women (Ng et al. 2014). Substantial variation was observed across age, sex, number of cigarettes per smoker per day and various countries. A recently published study evaluating participants (n=1,389) from Tasmanian Longitudinal Health Study cohort (TAHS) concluded that heavy maternal smoking (>20 cigarettes/day) during childhood increases the prevalence of post-bronchodilator (BD) airflow obstruction in the middle-age (9.3%; n=123). Maternal smoking was also associated with a 1.8-fold increase in the odds of airflow limitation for 7-year-old children, which was independent of gender and asthma severity. For the sub-group that was studied in middle age, heavy maternal smoking was associated with a 2.7-fold (95%CI: 1.3-5.7) increase in the odds of having post-BD airflow obstruction compared to individuals without exposure (Perret et al. 2016).

The association between current smoking and death from COPD is significantly higher than non-smokers among individuals >55 years (Men: relative risk= 27.8; 95%CI: 24.1-32.0; Women: relative risk= 25.0; 95%CI: 21.2-28.1). Among former smokers, the relative risk for mortality declined as the number of years since quitting increased (Carter et al. 2015). Moreover, the continuation of smoking is strongly associated with an increased rate of decline in lung function (FEV₁) over time, compared to ex-smokers or non-smokers (figure 1.5). This emphasizes the importance of smoking cessation for patients with chronic conditions such as COPD (Vestbo et al. 2011). In addition, second hand smoke exposure and children with smoking parents are also prone to development of airflow obstruction in non-smokers (Salvi & Barnes 2009). In smokers (>10 cigarettes/day), the decline in lung function can be much more rapid and frequently twice as great as in non-smokers (Rennard 1998). Evidence also highlights that active maternal smoking during pregnancy causes alterations in lung development and is responsible for both poor lung function and asthma in children (Gilliland et al. 2002).

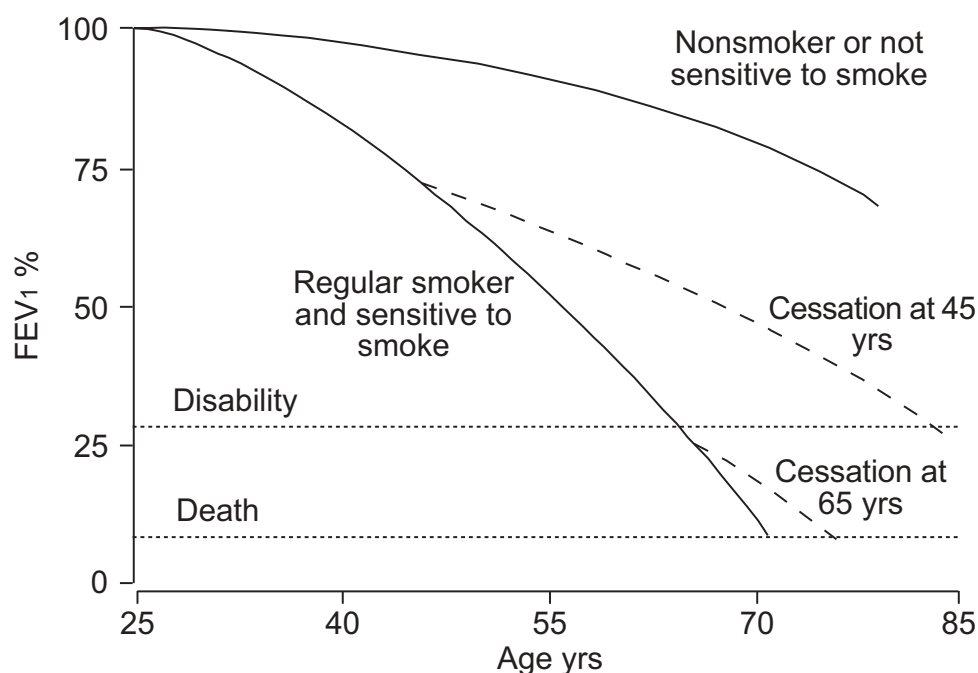


Figure 1.5. Decrease in forced expiratory volume in 1 second, according to smoking status.

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A number of potentially genetically controlled mechanisms have been suggested for explaining the role that cigarette smoke (CS) plays on lung tissue and parenchyma. Bronchial biopsies from mild to moderate COPD patients show significantly increased concentrations of CD8⁺ lymphocytes in proximal airways. The concentration of neutrophils in sputum also increases markedly (Keatings et al. 1996). CS mediates the production of inflammatory cytokines by directly stimulating epithelial cells and immune cells, particularly macrophages (Lim et al. 2000; Takizawa et al. 2001). CS appears to play an important role in the remodeling of the airways by interacting with epithelial cells, extracellular matrix and bronchial smooth muscle cells (Chung 2005). An imbalance in proteases/antiproteases is thought to lead to the destruction of compounds in the extracellular matrix, in particular elastin. A 40% alteration in the function of an antiprotease, α 1-antitrypsin (ATT), has been reported in smokers compared to nonsmokers (Gadek, Fells & Crystal 1979). In addition,

the upset in the oxidant (present in CS) and antioxidant (immune system) balance results in oxidative stress and is a likely factor for chronic inflammation (MacNee & Rahman 2001).

Validation of self-reported current smoking history by analyzing biomarkers associated with smoking increases the reliability and accuracy of both epidemiological and clinical analyses. We routinely performed exhaled CO measurements. Overall lifetime tobacco exposure is chiefly measured in pack-years and is frequently collected by surveys/questionnaires/personal interviews, and relies on the participants' responses (Florescu et al. 2009). Smoking habits vary greatly and are likely driven by changes in public health policies, as well as individual-associated factors such as occupational setup, socio-economic status (SES), and family-associated emotional incidents (e.g. death in family) (Hammond 2005). Inaccurate assessment of pack-years smoking may arise due to a number of reasons, such as poor recall of start age, failure of the participant to recall accurate exposure periods, false reporting, recall-bias, or loss of memory (Jaakkola & Jaakkola 1997). In addition, variable smoking habits for short periods are often either unreported or forgotten (England et al. 2007).

However, although debatable, there is evidence that self-reported smoking behavior during an individual's life-course is fairly reliable in most clinical studies (Kenkel, Lillard & Mathios 2003). Patrick and colleagues (Patrick et al. 1994) conducted a meta-analysis of published studies (n=26) and concluded that self-reports are mostly accurate, with an high average sensitivity (87.5%) and specificity (89.2%). The authors also recommend validation of current self-reported smoking status/duration by biochemical markers of tobacco exposure, such as cotinine, a major metabolite of nicotine (Al-Delaimy 2002; Kvalvik et al. 2012; Montalto & Wells 2007) or exhaled CO measures as performed by our research nurse.

Smoking cessation is the most effective and cost-effective intervention to prevent development and slow the progression of COPD. In terms of curbing the menace of tobacco smoking, Schauer and colleagues recently published that more than 50% of current smokers with a diagnosis of COPD were

offered cessation assistance counseling during their last clinic visit (Schauer et al. 2016). Healthcare professionals around the world should continue to provide tobacco cessation counseling, assistance and treatment to smokers with COPD in order to reduce the burden of smoking-related diseases, including COPD. The strategies for smoking cessation at a personal and community level are beyond the scope of this account, but a combination of limiting access (e.g. price), public media campaigns, quit facilities and potentially limiting supplies (e.g. Tobacco Free Generation legislation in Tasmania) are all important (Walters & Barnsley 2015).

1.3.1.2 Environmental exposure to noxious particles and gases (air pollution)

Although it is less potent than the smoking effect, high levels of air pollution have also been implicated as risk factors for the development of COPD. Approximately 25–45% of COPD patients are never-smokers (Salvi & Barnes 2009), in developing countries. Increasing population, urbanization, economic profile and pollution are a few of the factors and contribute substantially. The role of outdoor air pollution (including traffic-related fine particulate matter) as a causative factor for airflow limitation is gaining attention in recent times, due to increased vehicular pollution, as well as industrialization of the two most populated countries, China and India (Eisner et al. 2010).

Particulate matter with a diameter of 2.5 microns or less (PM_{2.5}), is considered to be the most health-damaging particles, as they could penetrate the deep lung tissue and initiate deleterious effects on the airway including but not limited to airway oxidative stress, pulmonary and systemic inflammation, ciliary dysfunction, amplification of infections, and increases in bronchial reactivity (Donaldson et al. 2002). The main components of PM₁₀ are sulfate, nitrates, ammonia, sodium chloride, black carbon, mineral dust and water (WHO, 2014).

Several investigators have assessed the effect of air pollution as a potential risk factor for COPD. In children and young adults, cross-sectional studies

have shown a relationship between higher outdoor pollutant levels (especially traffic related pollution) and lower lung function (Holguin et al. 2007; Janssen et al. 2003). Kulkarni et al. reported a likely causative dose-dependent inverse association between the carbon content (as a biomarker of particulate matter exposure, PM₁₀) of airway macrophages with lung function in children (Kulkarni et al. 2006). Furthermore, exposure to traffic-related pollution, characterized by the residential distance from a highway, was associated with impaired lung growth and lung function deficits at 18 years of age (Gauderman et al. 2007). Also, higher traffic density and proximity to highways was significantly associated with lower lung function (FEV₁) and FVC, but only in females (Kan et al. 2007), who are also at a greater risk of COPD than for those living farther away (Schikowski et al. 2005).

Long-term exposure to airborne particles and particulate matter is significantly associated with the risk of premature death and acute care hospitalizations, especially in patients with severe disease (Wordley, Walters & Ayres 1997; Zanobetti, Bind & Schwartz 2008). Moreover, daily variation in exposure to outdoor air pollution (mainly the particulate matter) significantly correlates with acute exacerbations of COPD (Sunyer 2001). The mechanisms that underlie obstruction due to air pollution are likely to be the same or/and similar to those due to cigarette smoking, but we do not yet have evidence for this, in spite of the importance of the issue.

1.3.1.3 Occupational exposure to dusts and fumes

Several longitudinal studies have shown an association between certain dusty occupational exposures and COPD, i.e., coal mining (Attfield 1985), gold mining (Holman et al. 1987), work related to tunnel-construction (Ulvestad et al. 2001), low levels of concrete dust containing crystalline silica exposure (concrete production industries) (Meijer, Kromhout & Heederik 2001), exposure to cotton in textile industries (Niven et al. 1997), workers exposed to welding fumes (Bradshaw et al. 1998), grain handlers and postal workers (exposure to endotoxins) (Schwartz et al. 1995) and animal feed industry (Post, Heederik & Houba 1998). In addition, exposures to chemical vapors,

irritants and fumes can also contribute to accelerated loss of airflow (Boschetto et al. 2006). One study involving railroad workers reported a positive association between COPD mortality and occupational exposure to diesel exhaust (Hart et al. 2006).

A population-based study found positive associations between several occupational exposure measures (mineral dusts, metal dusts and fumes, organic dusts, irritant gases or vapors, sensitizers, organic solvents, diesel exhaust, and environmental tobacco smoke) with COPD, among both ever-smokers and never-smokers (Weinmann et al. 2008).

Experimental studies in animal models have demonstrated that exposures to several agents, such as sulphur dioxide, mineral dusts, vanadium and endotoxin, are capable of inducing chronic obstructive bronchitis (Boschetto et al. 2006). Intratracheally instilled silica (quartz) produces airflow obstruction (functional change), which correlate with the presence of both emphysema and small-airway lesions (Wright et al. 1988). Inorganic dusts containing silica is associated with neutrophil and macrophage accumulation and morphological changes in rat lung. These morphological changes in small airways and lung parenchyma were similar to those in rats treated with elastase (Churg, Hobson & Wright 1989).

1.3.1.4 Biomass smoke inhalation

Various forms of biomass fuel (mostly animal dung, crop residues, and wood) are commonly used as sources of energy and constitute around 50% of total global household fuel consumption (cooking and heating in developing countries). Combustion of biomass fuel generates a large amount of smoke (>250 organic compounds, varying mainly by the type of fuel burnt and the combustion conditions) and adds to indoor and local air pollution (Naeher et al. 2007). Repeated and prolonged exposures to such biomass smoke with high emission factors (very low energy efficiencies; ~5–10%), while cooking or heating in poorly ventilated houses, have been associated with increased mortality and morbidity worldwide (de Koning, Smith & Last 1985). The

percentage of population using such solid fuels varies greatly among various countries and regions. It ranges from 77% in sub-Saharan Africa to 16% in Latin America and the Caribbean and in Central and Eastern Europe. In the majority of industrialized countries, solid biomass fuel use is below <5% (Rehfuess, Mehta & Prüss-Üstün 2006). A study of 12,000 slum dwellers from Pune (India) revealed an overall COPD prevalence of 6.5% (8.5% in males and 4.5% in females). Of those diagnosed with COPD, 69% were never smokers (Brashier 2005).

Hu et al. reviewed the available evidence up to 2010 in a meta-analysis and found a significant elevation in risk of developing COPD has been reported in people exposed to biomass smoke, compared with those without the exposure (OR: 2.44; 95% CI: 1.79-3.33). Biomass smoke was a significant risk factor for developing COPD in both males (OR: 4.30; 95%CI: 1.85-10.01) and females (OR: 2.73; 95%CI: 2.28-3.28) (Hu et al. 2010). Similarly, exposure to solid fuel (principally wood) smoke is consistently associated with COPD (OR: 2.80; 95%CI: 1.85-4.0) and chronic bronchitis (OR: 2.32; 95%CI: 1.92-2.80) (Kurmi et al. 2010). Exposure to biomass smoke from an early age is likely to result in impaired lung growth in later life. In a Nepalese study, the prevalence of airflow obstruction (FEV_1/FVC) in the biomass smoke-exposed group (20%) was twice that in the unexposed controls (11%) (Kurmi et al. 2013).

Animals exposed to wood smoke, at varying concentrations and times, showed reduced carbon monoxide diffusing capacity and increased airway resistance high-exposure group. Moreover, mild chronic inflammation and squamous metaplasia were also observed in the larynx of the wood-smoke exposed groups (Tesfaigzi et al. 2002). Similarly, guinea pigs subjected to wood smoke showed increased alveolar-capillary permeability, microvascular permeability, and histological injury scores in both airways and parenchymal tissues (Lin et al. 2001). In graded wood smoke exposure in Sheep, lung myeloperoxidase (MPO) activity was found to be higher (~19-fold) in smoke-exposed animal's lung tissue than in controls. A dose-dependent injury to

tracheobronchial epithelium and lung parenchyma in response to smoke was also reported (Park et al. 2004).

Women (and children) who spend most of their time indoors, especially in rural areas of developing countries, are exposed for 4-6 hours daily to higher concentrations of solid biomass fuel smoke that are produced by inefficient open fires. Ramírez-Venegas et al. reported that such women develop clinical characteristics of COPD, with reduced quality of life, and increased mortality comparable to that of tobacco smokers (Ramirez-Venegas et al. 2006), mainly characterized by greater small airway fibrosis (Rivera et al. 2008). The prevalence of COPD in non-smoking females living in rural areas in China was estimated to be three times higher than those living in urban areas (Liu et al. 2007). In a Mexican study, women exposed to biomass smoke had more air trapping but less frank emphysema than subjects who were exposed to tobacco smoke, implying an airway-predominant phenotype (Camp et al. 2014).

Better home ventilation and home design, and change from biomass to cleaner fuels is urgently needed in order to reduce biomass smoke-induced mortality and morbidity. Furthermore steps should be taken to increase awareness about the health effects of biomass fuel smoke inhalation among physicians and health administrators, which may improve diagnosis and treatment of affected patients and trigger preventive actions through education, research, and policy change (Silva, Oyarzun & Olloquequi 2015).

1.3.2 Host factors

1.3.2.1 Age

Global demographic projections suggest that the proportion of population over the age of 60 years will double from around 11% in 2000 to 22% in 2050, with the absolute number of people >60 years estimated to increase from 605 million to 2 billion by 2050. More than 80% of this demographic group will live in less-developed countries, compared with about two-thirds at present (WHO

2014). Ageing is associated with a progressive degeneration of the lung tissues, which has a negative impact on the structure and function of lungs, and is an independent predictor for COPD (Decramer & Rossi 2001). These inherent changes coupled with other risk factors (exposures) may potentially lead to symptomatic airflow limitation. Thus, COPD is reported to be three-times higher in the elderly (>60 years), than other age groups (Fukuchi et al. 2004). In an international multisite survey, the overall prevalence of COPD was approximately 10% for people >40 years, nearly doubling with every 10-year increment of age, and so estimated to reach 19–47% for men and 6–33% for women aged 70 years and older (Buist et al. 2007). In individuals over 60 years, COPD is the third-most burdensome disease after ischaemic heart disease (IHD) and stroke, accounting for 43.3 million disability adjusted life years (DALYs) globally, with again 86% of this estimated burden arises in under-developed regions ('Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013' 2015).

The process of ageing also has profound effects on the cellular, organ and organismal level. The aging-associated inflammation/structural change is the results of several factors, such as failure of reactive oxygen species (ROS) elimination, impaired repair of damaged DNA, reduction in anti-ageing molecules (histone deacetylases and sirtuins) and/or stimulation of pro-ageing molecules and telomere shortening (Ito & Barnes 2009). Natural ageing of the lung is also associated with dilatation of alveoli with an enlargement of airspaces and decrease in gas exchange surface area, together with a loss of supporting tissue for peripheral airways (termed as “senile emphysema”), resulting in decreased static elastic recoil of the lung and increased residual volume and functional residual capacity (Janssens, Pache & Nicod 1999). However the changes in structure and functional characteristics caused by isolated airspace enlargement seen in the elderly lung (>60 years) could be differentiated from pathological (smoking-related) emphysema by the absence of alveolar wall destruction (Verbeken et al. 1992).

Functionally, vital capacity is reduced with ageing, although the total lung capacity remains unchanged (Chan & Welsh 1998). Respiratory muscle strength has been shown to decrease in 65 to 85-year-old healthy males. Thus, diaphragm strength is reduced by approximately 25% in healthy elderly individuals compared with young adults (Enright et al. 1994). There is a decline in the diffusing capacity of the healthy nonsmoker lung for carbon monoxide (DL_{CO}) of 2.03 mL/min/mm Hg per decade from middle age onward for men and a decrease of 1.47 mL/min/mm Hg per decade for women (Neas & Schwartz 1996). A progressive decline in maximum oxygen consumption (Vo₂ max) also occurs with advancing age, principally due to the loss of muscle mass and respiratory limitations with senescence (Fleg & Lakatta 1988).

1.3.2.2 Genetic Factor

Although inconsistent, several population studies have suggested a shared genetic risk of COPD. The strongest genetic risk factor is the hereditary deficiency of α 1-antitrypsin (ATT, encoded by *SERPINA1*) (Stoller & Aboussouan 2005), which accounts for around 5% of cases of COPD, yet only a minority (~4%–5%) of ATT-deficient individuals are identified (Lieberman, Winter & Sastre 1986), that too at a delay of around 5 to 10 years (Stoller et al. 2005). Large-scale genome-wide studies (GWAS) have identified key polymorphisms linked to COPD, including a-nicotinic acetylcholine receptor (*CHRNA 3/5*), *TNS1*, *GSTCD*, *HTR4*, *AGER* and *THSD4* (Pillai et al. 2009; Repapi et al. 2010; Soler Artigas et al. 2011). In a recently published meta-analysis of COPD, Cho and coworkers (Cho et al. 2014) confirmed associations at three known loci and found additional genome-wide significant associations with moderate-to-severe COPD near *RIN3* and with severe COPD near *MMP12* and *TGFB2*.

Data from current and former smokers participating in the COPDGene Study indicate that subjects with mild airflow obstruction showed substantial clinical heterogeneity, with *FAM13A* and *BICD1* associated with emphysematous subjects and *HHIP* and *CHRNA3* associated with the lowest FEV₁ %predicted

cluster (Lee et al. 2014). The recent advances in GWAS in identifying the genomic regions associated with COPD have certainly improved our understanding. Given the large differences in genetic background of different ethnic populations, fairly large-scale studies in genetically diverse populations are warranted.

1.3.2.3 Gender

Historically, COPD has been considered as a disease of males who started smoking several decades before women. Indeed, growing epidemiologic data since late 90s suggest that females may be more susceptible than males to the effects of cigarette smoking and development of COPD. The interaction of gender and smoking on development of COPD was assessed in two population studies, the Copenhagen City Heart Study (CCHS) and the Glostrup Population Studies (GPS) (n=13,897). The participants were followed for 7-16 yrs. Smoking had greater impact on the lung function of females than males in both the studies. Moreover, the risk of hospitalization for COPD was higher in females than in males (Prescott et al. 1997).

Among current smokers, males and females differ in their susceptibility to developing COPD. In a systematic review and a meta-analysis (11 studies; n=55,709), Gan et al. presented additional evidence that smoking women have more decline in lung function, especially over the age of 45–50 years (Gan et al. 2006), 0.98%/pack-year in men vs. 1.21%/pack-year for women (Dransfield et al. 2006). In another similar study of COPD patients, Sorheim et al. reported that women had a lower FEV₁ (48.7% predicted for women vs. 55.8% predicted for men) and higher prevalence of GOLD COPD stage (III and IV) in both the low and high smoke exposure subgroup (Sørheim et al. 2010). Again, with a different approach in a longitudinal study of COPD patients participating in the Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints (ECLIPSE) study (n=1928), the annual decline in CT lung density was more rapid in women than in men and in current smokers (additional-0.29 [SE 0.14] g/L per year) than in former smokers (Coxson et al. 2013).

Females also report more symptoms, such as dyspnea, depression and lower health-related quality of life, and appear to have an airway predominant disease phenotype (Allen-Ramey, Gupta & DiBonaventura 2012; Martinez et al. 2007).

The effects of hormones on COPD are still largely unknown. One study demonstrated no difference in the incidence of COPD in women on hormone replacement therapy (Barr et al. 2004), while another study showed that hormone replacement therapy did significantly improve lung function in post-menopausal women (Carlson et al. 2001). Although its mechanisms are yet to be fully elucidated, it is likely to be a dose response as women are smaller in size and their lungs are subjected to a greater stress for the same 20 cigarettes/day compared to men (Prescott et al. 1997). The notable sex disparity in COPD may have potential diagnostic, therapeutic and prognostic implications and are crucial for effective management of female patients with airway diseases.

1.3.2.4 Asthma (bronchial hyper-responsiveness, BHR)

Many individuals share characteristics of both asthma and COPD, and have largely excluded from pharmaceutical studies. This Asthma-COPD overlap syndrome (ACOS) occurs in patients with fixed airways obstruction characteristic of COPD and presence of typical symptoms and features associated with asthma (eosinophilic bronchial and systemic inflammation, increased reversibility of airflow obstruction, and increased response to inhaled corticosteroids). In terms of prevalence of ACOS, Vonk et al. studied 228 adults (13-44 years at baseline) who were followed up for 26 years, and reported that approximately 16% of asthmatic patients developed fixed airway obstruction (reduced FEV₁, and poor reversibility of airway obstruction), as well as symptoms characteristic of COPD (cough, phlegm, dyspnoea) at follow (Vonk et al. 2003).

Compared to COPD individuals, patients with ACOS have more rapid disease progression and experience more frequent exacerbations as well as more wheezing and dyspnoea (Barrecheuren, Esquinas & Miravittles 2015). Clinically, asthmatics with fixed airflow obstruction have distinct clinical characteristics compared with patients with smoking-related COPD, and have significantly more eosinophils in peripheral blood, sputum, bronchoalveolar lavage, and airway mucosa; fewer neutrophils in sputum and bronchoalveolar lavage fluid; a higher CD4+/CD8+ ratio of T cells infiltrating the airway mucosa; and a thicker reticular layer of the epithelial basement membrane (Fabbri et al. 2003). In a 5 years follow-up study, patients with COPD (n=21) and asthmatics with irreversible airflow obstruction (n=16) had a greater rate of lung function decline as well as significantly increased exacerbation rates, compared to the asthma group with fully reversible airflow obstruction (n=15) (Contoli et al. 2010). Data from our group (n=1,389) from the population-based Tasmanian Longitudinal Health Study (TAHS), shows that both active smoking and current clinical asthma contribute substantially to fixed airflow obstruction in middle age (overall prevalence: 6.0%; 95%CI: 4.5–7.5%), especially among those with atopy (Perret et al. 2013).

1.3.2.5 History of respiratory infections (Pneumonia and Tuberculosis)

Childhood lower respiratory tract infections (LTRIs), especially tuberculosis (TB) and pneumonia, could damage the respiratory tract and possibly lower lung function permanently. With increasing age it may lead to accelerated decline in airflow and increase the risk for COPD (Svanes et al. 2010). The European Community Respiratory Health Survey assessed a total of 9,175 individuals (20-44 years) and concluded that childhood serious respiratory infections potentially have a detrimental effect on adulthood lung function (FEV₁/FVC ratio: -1.2%; 95%CI: -1.8 to -0.6), and could influence development and persistence of adult respiratory morbidity (Dharmage et al. 2009).

We have recently published a review on the overlap of TB and COPD, in which we discussed the increased risk of developing airflow obstruction characteristic of COPD, post successful treatment of TB (O'Toole, Shukla &

Walters 2015). It is suggested that active TB disease can lead to localised bronchial fibrosis, and/or parenchymal destruction (emphysema), with loss of elastic recoil (Jordan, Spencer & Davies 2010). However, TB itself, if treated early, does not alter the major multiplicative effect with smoking on COPD.

In addition to TB, epidemiological evidence exists suggesting an association between childhood pneumonia and obstruction (loss of lung function) in adult life. This certainly raises the question of whether childhood pneumonia is a risk factor COPD. As a part of United States COPD Gene Study, Hayden and colleagues collected data from 10,192 individuals (45-80 years) and concluded that individuals who reported pneumonia during childhood are at greater risk of smoking-related COPD, chronic bronchitis, frequent episodes of COPD exacerbations, and lower lung function (OR: 1.40; 95%CI: 1.17-1.66) (Hayden et al. 2015). Recently published data from a longitudinal study as a part of Tucson Children's Respiratory Study in 44 subjects, the authors conclude that radiographically diagnosed pneumonia before the age of 3 years was associated with small but persistent lung function deficits (mean: -2.5%; SE: -0.8% in post-bronchodilator FEV₁/FVC) up to age 26. Other lower respiratory infections (n=294) at an early age were also associated with very mild loss of lung function (mean: -1.1%; SE: -0.5%; post-bronchodilator FEV₁/FVC) (Chan et al. 2015). Recent statistical data from our group (n=1,389, TAHS study) suggest that childhood measles significantly worsens the adverse association of having current asthma and smoking on chronic airflow limitation in the middle age. Moreover, childhood pneumonia was associated with reductions in adult total lung capacity (Jennifer et al. 2014).

1.3.2.6 Socioeconomic status (SES)

Socioeconomic status (SES), commonly measured by education, occupation, and/or income has a profound impact on health and longevity. Once COPD has developed, SES may also influence the prognosis of the disease (Miravittles et al. 2011; Pena et al. 2000). Various factors such as differences in COPD-treatment, varying smoking habits, unemployment rates, lack of

social relations and living alone, are frequently present in individuals with low SES and could negatively influence prognosis (Lange et al. 2014).

A social class gradient in the prevalence of respiratory symptoms in adults (cough and sputum) was first reported in early studies in the UK (Speizer & Tager 1979). In a study conducted in two British towns based on mortality rates from respiratory diseases (high mortality: Caerphilly; Bath: low mortality), the overall mean FEV₁ and FVC was found to be significantly higher in Bath than Caerphilly (Burr & Holliday 1987). Individuals at the lowest SES are twice as likely to have poor COPD outcomes compared to those of the highest SES. In addition, there is a significant negative correlation between lung function (FEV₁ reductions of >300 mL in men and >200 mL in women) and SES (Hegewald & Crapo 2007). Yin et al. showed that subjects with low educational level had a significantly increased risk of COPD. Similarly, increased risk was observed for household income and COPD (OR: 1.64; 95%CI: 1.28-2.09), but only in urban setting (Yin et al. 2011).

In Victoria (Australia), subjects living in the most disadvantaged areas had a significantly greater COPD burden ($r=-0.39$), disease readmissions ($r=-0.49$) and mortality ($r=-0.51$). Moreover, Gershon et al. found that COPD-mortality declined faster in people with the highest SES (38%) compared with the lowest SES (30%), causing increased disparity between rich and poor (Gershon et al. 2014).

1.3.2.7 Nutrition

Different dietary factors (dietary-patterns, foods, nutrients, vitamin D etc.) have been associated with the respiratory function in COPD along with complex interplays between environmental and genetic factors (Berthon & Wood 2015). Several studies have reported that fruits and vegetables exert beneficial effects on lung health (Keranis et al. 2010). Dietary fiber intake was also independently associated with higher pulmonary function and reduced prevalence of COPD in a study conducted in the USA (Kan et al. 2008). Moreover, frequent consumption of cured meats (high in nitrites) was

associated with higher prevalence and incidence of COPD among men in the USA (Varraso et al. 2007). It has also been suggested that subjects deficient in certain nutrients such as magnesium, omega(ω)-3 fatty acids, and lower fish intake had lower FEV₁ than those who had sufficient nutrient intake (Britton et al. 1994; Shahar et al. 1994). Epidemiological studies have shown promising associations between vitamin D and lung health. Foong and Zosky reviewed the role of vitamin D deficiency in disease onset, progression and exacerbation of COPD, and concluded that vitamin D plays a key role in the immune function and/or lung growth, although only reversing vitamin D deficiency is promising (Martineau et al. 2015). The effect of vitamin D on microbe-induced exacerbations is less clear (Foong & Zosky 2013). Holistic management for these patients should be revisited, which could include nutritional modulation. Moreover, metabolic syndrome must be considered in the management of COPD, with recent evidence indicating that obesity itself can increase BHR (Byrne et al. 2015).

Chapter 2

Respiratory infections in smokers and COPD

2.1 Cigarette smoke (CS) exposure and risk of respiratory infections

2.1.1 Components of CS

CS is recognized as a crucial factor for increased risk of respiratory infections, as well as host immunity in healthy individuals. Various ingredients, including the tobacco blend, the cigarette paper, the type and efficiency of the filter, and the degree of tip ventilation determine the chemical composition of cigarette smoke. More than 4,500 different substances have been identified in fresh CS. Chemical entities include neutral gases, carbon oxides, nitrogen oxides, amides, imides, lactames, carboxylic acids, lactones, esters, aldehydes, ketones, alcohols, phenols, amines, volatiles N-nitrosamines, N-heterocycles, hydrocarbons, nitriles, anhydrides, carbohydrates, ethers, nitro-compounds, metals and short- and long-living radicals. The quantities of the components in the mainstream smoke of a single cigarette vary greatly (Borgerding & Klus 2005).

In addition to chemicals, the bacterial metagenomic data from a cigarette-based study revealed 15 different classes of bacteria and a broad range of potential pathogens in >90% of all cigarette samples (*Acinetobacter*, *Bacillus*, *Burkholderia*, *Clostridium*, *Klebsiella*, *Pseudomonas aeruginosa*, *Serratia*, *Campylobacter*, *Enterococcus*, *Proteus* and *Staphylococcus*) (Sapkota, Berger & Vogel 2010). Although several studies have documented the presence of microbial toxins (both bacterial and fungal), the risk of infection by potential pathogens from inhaling the burnt CS remains unclear (Larsson et al. 2008).

Inhaled particulate matter from CS is deposited in the respiratory tract in a particle-size dependent manner. Larger particles (diameters >0.1 µm) are deposited in the upper and larger airways, and smaller particles (<0.1 µm) penetrating deep into the alveolar spaces, as well as more rapid uptake into

cells, and into circulation (Oberdorster et al. 2002). Impaired clearance of this particulate matter causes particle retention in lung tissues, resulting in a chronic, low-grade inflammation that may be important in the progression of chronic lung diseases associated with long-term smoking (Ling & van Eeden 2009).

2.1.2 CS exposure and risk of LRTI

Lower airway bacterial colonization (assessed in BAL sample) was found in approximately 60% of asymptomatic smokers (Qvarfordt et al. 2000). Bacterial colonization of the human respiratory mucosal surface is a dynamic process in which bacteria are acquired, replaced, and reacquired many times in a lifetime. Data from both prospective and retrospective studies have shown increased bacterial and viral infections among cigarette smokers compared with non-smokers (Sherman 1992). In a study of 867 individuals (both males and females), the authors found that smoking significantly increases the likelihood of having a lower respiratory tract illness, as well as severity of acute respiratory tract infections by bacterial pathogens when they occur, resulting in morbidity and lost productivity (Aronson et al. 1982). A recent study also showed that current smoking status is associated with a higher mortality from various infections (relative risk: 2.3; 95%CI: 2.0-2.7) (Carter et al. 2015).

Bensenor et al. reported an increased risk of prolonged 'colds' in women who were heavy smokers, while nonsmoking women passively exposed to CS had both more frequent and longer duration of such colds (Bensenor et al. 2001). As documented extensively, the most frequent microorganisms isolated from the lower respiratory tract of smokers and of persistently colonized patients are non-typeable *H. influenzae* (NTHi), *S. pneumoniae* and *P. aeruginosa* (the latter only in more severe stages of COPD). Furthermore, NTHi is also the most frequently isolated bacterial pathogen from patients with acute exacerbations of COPD (Sethi & Murphy 2008).

Zalacain et al. found current smoking to be significantly associated (OR: 3.17; 95%CI: 2.50–8.00) with pulmonary infection with pathogenic bacteria, with *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* being the most frequently cultured species (Zalacain et al. 1999). Exposure to cigarette smoke also increased invasive pneumococcal infections in adult smokers (Nuorti et al. 2000). Further cross-sectional surveillance study of nasopharyngeal and oropharyngeal carriage of *S. pneumoniae* and *H. influenzae* in 208 children aged <60 months and their mothers found higher *S. pneumoniae* carriage rate not only in actively smoking mothers, but also the children exposed to CS, compared to nonsmoking mothers and non-exposed children respectively. However, smoking or exposure to smoke did not increase upper airway *H. influenzae* carriage rates either in children or mothers (Greenberg et al. 2006).

Gaschler et al. observed increased pulmonary inflammation and lung damage in CS-exposed NTHi-challenged mice as compared with control NTHi-challenged mice. The authors reported that NTHi-challenge in CS-exposed mice resulted in a noticeable upregulation of the inflammatory mediators, such as monocyte chemotactic protein (MCP)-1, MCP-3, MCP-5, interferon gamma-induced protein (IP)-10, and macrophage inflammatory protein (MIP)-1 γ , while control NTHi-challenged mice showed a different profile of elevated levels of tumor necrosis factor (TNF)- α , interleukin (IL)-6, (MIP-2), and KC/GRO α (Gaschler et al. 2009).

The effect of tobacco smoking on the occurrence of pneumonia in the adult community was provided by a population-based case-control study (controls: 475; patients: 205) in Barcelona (Spain). The authors found that smoking any type of tobacco had an increased risk of CAP for **ever-smokers** (OR: 2.0; 95%CI: 1.24-3.24), **current smokers** (OR: 1.88; 95%CI: 1.11-3.19); and **ex-smokers** (OR: 2.14; 95%CI: 1.26-3.65). A positive trend for increased risk of CAP was observed for an increase in the duration of the habit, the average number of cigarettes smoked daily, and cumulative cigarette consumption. This shows that giving-up smoking is still a big risk factor, though former smokers did have 50% reduction in the OR 5 years after the cessation of smoking (Almirall et al. 1999). In a prospective study during the period of 1997

to 2011 (n=1387), current smoking was also significantly associated with elevated risk of invasive pneumococcal disease, even in young adults (Grau et al. 2014). In another multicenter, prospective cohort study (n=4,288), Bello et al. reported that current smokers with pneumococcal community acquired pneumonia (CAP) often developed severe sepsis and require hospitalization at a younger age, despite fewer comorbid conditions. In consequence, continued smoking increased the risk of 30-day mortality independent of tobacco-related comorbidity, age, and other comorbid conditions (Bello et al. 2014).

The relative risk of development of tuberculosis for heavy smokers compared with nonsmokers is also significantly higher (RR: 2.17; 95%CI: 1.29-3.63) (Yu, Hsieh & Peng 1988). In a case-control study of the smoking habits of 27,000 urban and 16,000 rural men in India, Gajalakshmi et al. found higher tuberculosis prevalence risk ratio 2.9 (95%CI; 2.6-3.3) for ever-smokers compared with never-smokers. The authors also found that the smoking attributable fraction of deaths from tuberculosis was 61%, which was more than smoking-attributable deaths from vascular disease or cancer (Gajalakshmi et al. 2003).

A number of studies have established that CS is also associated with significantly increased risk of pulmonary viral infections. Using *in-vivo* mice models, it has been shown that CS indeed affects inflammatory processes, viral clearance and secondary immune protection post-influenza virus infection (Gualano et al. 2008). It has been suggested that CS mainly affects primary antiviral inflammatory processes, whereas secondary immune protection remains intact (Robbins et al. 2006). Moreover, smokers exhibited higher incidence of non-acute viral infection (clinical 'cold') than nonsmokers (OR: 2.23; 95%CI: 1.03-4.82), to specific viruses (Rhinovirus, Respiratory Syncytial Virus or Coronavirus) (Cohen et al. 1993).

2.1.3 Second-hand smoke (SHS) exposure and risk of LRTI

There is robust evidence that exposure to SHS has similar (although quantitatively less) adverse effects to active smoking on lung infections, both in children (Kum-Nji, Meloy & Herrod 2006; Nandi et al. 2001) and adults (Jaakkola & Jaakkola 2002). Chronic exposure of mice to SHS resulted in significantly higher loads in the lungs, of both NTHi and *S. pneumoniae*, two most common respiratory bacterial pathogens (Voss et al. 2015). One study (n=46 followed up to 14 weeks) found that the exposure to SHS increases the incidence of chronic respiratory symptoms, such as sneezing, sore throat and cough in non-smoking adults (Segala et al. 2004).

A recent *in vitro* study reported reduced phagocytic activity of murine macrophages pretreated with SHS (and direct CS), which resulted in increased bacterial (*P. aeruginosa*) survival, compared to cells exposed to room-air control, possibly due to reduced phagocytosis by AMs (Ni, Ji & Vij 2015).

Although public health campaigns have focused at increasing the awareness of the dangers of SHS, and many jurisdictions worldwide have enacted legislation to restrict smoking in public places and at work, a large global population is frequently exposed to SHS, and this urgently needs to be better addressed.

2.1.4 Mechanisms

Several mechanistic studies at molecular and cellular level on immunosuppressive effects of CS have been reported, mainly focusing on impairment of host immune system in healthy individuals. Mehta and colleagues (Mehta, Nazzal & Sadikot 2008) reviewed the immunosuppressive effects of CS and the mechanisms by which smoking affects host innate immunity including structural and functional changes in the respiratory tract (figure 2.1).

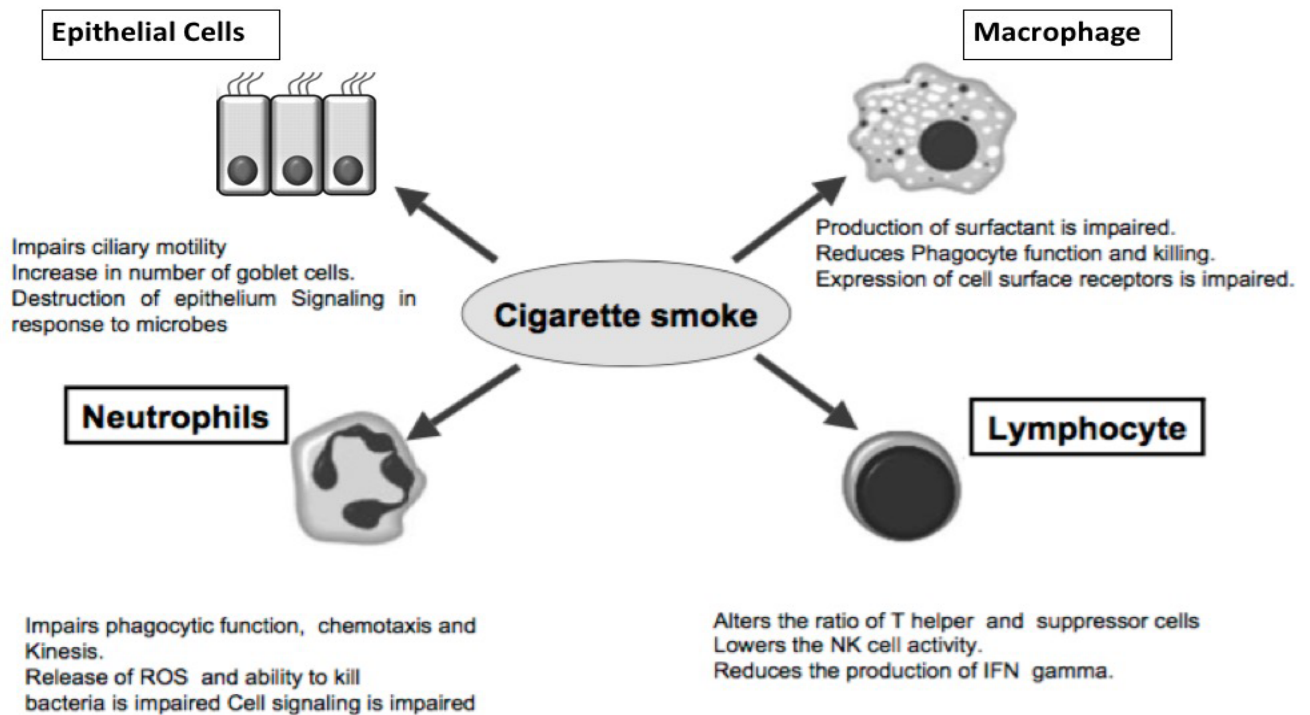


Figure 2. 1 The effects of smoking on epithelial, macrophage, neutrophils and lymphocytes.

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The specific mechanisms by which CS increases the risk of respiratory infections include structural changes in the respiratory tract and an impaired host-immune response. Exposure to CS results in structural changes in the respiratory airways, including luminal inflammation and wall fibrosis, increased mucosal permeability (disruption of tight junctions), impairment of the mucociliary clearance, and changes in pathogen adherence (Burns et al. 1989; Dye & Adler 1994). CS also induces direct oxidative damage to membrane lipids and causes extensive DNA breaks, triggering repair and apoptotic cascades (Kim et al. 2004). Maunders et al. assessed the effects of cigarette smoke (determined at 1, 6, and 24 h post-exposure) on gene-expression profiles associated with cell signaling and function in a three-dimensional air-liquid interface model of tracheobronchial epithelium, which was grown from primary human lung epithelial cells. Genes associated with cellular adhesion decreased, and antioxidant and detoxifying genes were activated (Maunders et al. 2007). CS has been shown to decrease *M.*

catarrhalis-induced human β -defensin-2 (hBD-2) antimicrobial peptide expression and prostaglandin E2 induction, resulting in increased bacterial load on the bronchial epithelium of smokers (Zhang et al. 2011).

Bronchoalveolar lavage fluid (BAL) studies have demonstrated a smoking-dependent decrease in the absolute number of CD4⁺ cells, and an increase in CD8⁺ cells with a lower CD4⁺/CD8⁺ cell ratio in moderate smokers compared to nonsmokers, suggesting a deficit in cell-mediated immunity in the lung alveoli, which is the first-line defense against infection (Costabel et al. 1986). The retention of CD8⁺ T cells in the lungs may activate alveolar macrophages (AMs) to produce matrix metalloproteinase 12 (MMP-12), a potent elastin-degrading enzyme that has been linked to emphysema (Hautamaki et al. 1997). CS has also been shown to weaken the defensive ability of virus-specific CD8⁺ memory effector T-cells in mice lung (Gualano et al. 2008).

Although, CS increases the number of alveolar macrophages (AMs), it also activates them to produce pro-inflammatory mediators, reactive oxygen species and proteolytic enzymes, resulting in inflammation and tissue damage, which may make it more difficult to clear microbial pathogens (Sopori et al. 1998). It compromises the ability of AMs to phagocytose bacteria and apoptotic cells, as well as its ability to sense pathogen-associated molecular patterns (PAMPs) (Berenson et al. 2006; Gaschler et al. 2008; Hodge et al. 2007). CS exposure of alveolar macrophages (AMs) is shown to result in impaired NTHi ingestion, although not the ingestion of inert particles. Phosphoinositide 3-kinase (PI3K) signaling, including Akt phosphorylation, is essential for NTHi phagocytosis by AMs, and AMs exposed to CS show diminished phospho-Akt levels, which may account for sub-optimal phagocytic ability. Similar observations were made using immortalised macrophages and BAL macrophages from both smokers and COPD patients, compared with macrophages from never-smokers (Martí-Llitas et al. 2009).

In terms of impaired pulmonary Pneumococcal clearance, CS has been shown to prevent complement-mediated phagocytosis of *S. pneumoniae* by

AMs in mice exposed to CS for 5 weeks followed by intratracheal bacterial challenge, although the ingestion of unopsonised bacteria or IgG-coated microspheres was not affected (Phipps et al. 2010).

Woodruff et al. reported a distinctive state of activation of AMs in smokers that distinguished them from those in non-smokers (Woodruff et al. 2005). CS may induce phenotypic switching in macrophage population. Thus, CS may induce partial M1 deactivation or partial M2 activation of macrophages. The balance and intensity of this skewing has direct implications for the immune system and its response to disease because effective host defense requires a macrophage activation programme that is appropriate for the particular type of pathogen. The release of cytokines (e.g. $\text{TNF}\alpha$, IL-1, IL-2 and IL-6) from macrophages may also be altered in smokers (McCrea, KA et al. 1994), which in turn could suppress neutrophil chemotaxis and migration (Corberand et al. 1979). Several studies have also shown decreased natural killer (NK) cell numbers and activity in smokers compared with non-smokers (Swann et al. 2007). Exposure to cigarette smoke attenuates the cytotoxic activity and cytokine production of NK cells in both humans and mice models (Mian et al. 2008).

Another important aspect of CS exposure is upregulation of microbial receptors on respiratory epithelial, which have been a focus of my studies. This interest in our group started with the observation that exposure to cigarette smoke extract (CSE) induces increased expression of pneumococcal adhesion receptor (PAFr) in lower airway epithelial cells in culture, as well as in living mice, which correlated with enhanced adhesion of *S. pneumoniae* (Grigg et al. 2012). There was also a suggestion of upregulation of PAFr on normal-smoker's epithelium in biopsy material.

2.1.5 Does cigarette smoke affect the respiratory Microbiome?

Smoking-related changes in the lung microbiome could be relevant for disease progression and exacerbations in a number of pulmonary diseases, including smoking-related COPD. But data on these issues are limited.

Results from a multicenter cohort study (n=64), based on modern culture-independent techniques such as RT-qPCR (using 16S-rRNA) show that the oro-pharyngeal microbiome (oral wash) differed in nonsmokers and smokers, but no difference was found in lung microbial communities using BAL (Morris et al. 2013). Another study found that the nasopharyngeal microflora of smokers (n=20) contained more potential pathogens compared with those of non-smokers (n=20), especially *S. pneumoniae*, *H. influenzae*, *Moraxella catarrhalis*, and *S. pyogenes* (Brook & Gobeil 2005). The authors also noted fewer aerobic and anaerobic organisms with interfering capabilities against colonization by pathogenic bacteria.

2.2 Respiratory infections in patients with COPD

In the 1950s and 1960s, British investigators developed a hypothesis suggesting that recurrent bronchial infections were the primary reason in smokers for development of progressive airways obstruction (Fletcher 1959). Data from The Copenhagen City Heart Study (n=9435; men and women aged between 30 to 79 years, followed up to 10 years) suggested that smokers with chronic cough and sputum exhibited more accelerated decline in lung function (FEV₁) than asymptomatic individuals. In the same study, chronic mucus hypersecretion was also associated with subsequent hospitalization due to COPD for both genders (Vestbo, Prescott & Lange 1996). Another study (n=30; followed up to 12 months) suggested a link between lung function decline and both bacterial counts and greater airway inflammation in sputum, as assessed by IL-8 levels. The authors found greater FEV₁ decline in subjects who exhibited a change in the colonizing bacterial type compared with those with persistence of a single bacterial species over 12 months (Wilkinson et al. 2003).

In the past two decades, our understanding of the pathogenesis of airway infections has increased substantially. In particular, novel molecular, cellular, and immunologic techniques used to study the host–pathogen interaction have been applied in assessing the role of microbial infections in COPD. It is also now acknowledged that airway infection is the predominant cause of exacerbations, which then contributes to the natural history of COPD. The prevalence and role of various microbial pathogens in both stable stages and exacerbations of COPD has been reviewed by Sethi and Murphy (Table 2) (Sethi & Murphy 2008).

Table 2. Microbial pathogens in COPD. *Reproduced with permission from Sethi & Murphy (2008). Copyright Massachusetts Medical Society.*

Microbe	Role in Exacerbations	Role in Stable Disease
Bacteria		
<i>Haemophilus influenzae</i>	20–30% of exacerbations	Major role
<i>Streptococcus pneumoniae</i> (<i>Pneumococcus</i>)	10–15% of exacerbations	Likely, still being studied (Domenech et al. 2012; Karin et al. 2015)
<i>Moraxella catarrhalis</i>	10–15% of exacerbations	Minor role
<i>Pseudomonas aeruginosa</i>	5–10% of exacerbations, prevalent in advanced disease	Probably important in advanced disease
Enterobacteriaceae	Isolated in advanced disease, pathogenic significance undefined	Undefined
<i>H. haemolyticus</i>	Isolated frequently (misdiagnosed), unlikely cause	Unlikely
<i>H. parainfluenzae</i>	Isolated frequently, unlikely cause	Unlikely
<i>Staphylococcus aureus</i>	Isolated frequently, unlikely cause	Unlikely
Viruses		
Rhinovirus	20–25% of exacerbations	Unlikely
Parainfluenza virus	5–10% of exacerbations	Unlikely
Influenza virus	5–10% of exacerbations	Unlikely
Respiratory syncytial virus	5–10% of exacerbations	Controversial
Coronavirus	5–10% of exacerbations	Unlikely
Adenovirus	3–5% of exacerbations	Latent infection described, pathogenic significance undefined/unlikely
Human metapneumovirus	3–5% of exacerbations	Unlikely
Atypical bacteria		
<i>Chlamydophila pneumoniae</i>	3–5% of exacerbations	Commonly detected, pathogenic significance undefined
<i>Mycoplasma pneumoniae</i>	1–2% of exacerbations	Unlikely
Fungi		
<i>Pneumocystis jiroveci</i>	Undefined	Commonly detected, pathogenic significance undefined/unlikely

2.2.1 Microbial colonization/infections in stable COPD

Thus, persistence of bacteria in the lower respiratory tract, especially with NTHi in relatively stable COPD patients is characteristic of the disease, and is termed 'colonization', although that may well be a misnomer that underestimates the importance of what is in reality chronic infection (Robinson 2004). Previously, 'healthy' lungs were considered relatively uninhabitable for microorganisms, especially bacteria, at least based on culture-detection techniques. Novel molecular techniques have recognized a wide range of bacteria that are likely to update our understanding of the role of microorganisms in the pathogenesis of COPD. It has been reported that lower respiratory tract is colonized by a 'microbiome' even in healthy individuals. One study reported that COPD is not associated with an alteration of the respiratory microbiome, whereas others have reported changes in relative abundance of specific microbial phyla and in microbial diversity (Cabrera-Rubio et al. 2012; Erb-Downward et al. 2011; Han et al. 2012; Pragman et al. 2012; Sze et al. 2012). In COPD patients; potentially pathogenic microorganisms (PPM) are culturable in lower airway fluids (sputum and bronchoalveolar lavage) in up to 65% of patients at any one time (Banerjee, Khair & Honeybourne 2004; Cabello et al. 1997; Garcha et al. 2012; Soler et al. 1999). Traditionally, this has been from an inadequate innate immune response of the host leading to airway bacterial colonization and subsequent invasion of the host tissue, i.e., overt bacterial infection (Hill et al. 2000; Sethi et al. 2006; Sethi et al. 2004; Weinreich & Korsgaard 2008). Moreover, qPCR has proven to be advantageous as it identifies a significant number of patients with potentially pathogenic bacteria as well as airway inflammation in COPD, which are not identified by traditional culture-based methods (Bafadhel et al. 2015).

COPD patients whose respiratory tracts are 'colonized' with bacteria experience increased daily respiratory symptoms (breathlessness, cough and sputum), an accelerated decline in lung function, increased airway and systemic inflammation, and impaired health related quality of life (Desai et al.

2014; Marin et al. 2012; Wilkinson et al. 2003). Thus, this is not a benign condition.

The most common “colonizers” in the stable state of COPD are non-typeable *Haemophilus influenzae* (NTHi) (~60% of patients), *Moraxella catarrhalis* (~48% of patients) and *Streptococcus pneumonia* (~28% of patients) (Bafadhel et al. 2015; Barker et al. 2015). Moreover, bacterial colonization of the respiratory tract in COPD patients is believed to be dynamic, as the bacterial flora frequently changes in terms of both strains and species, but again predominantly with the above-mentioned bacteria (Marin et al. 2010; Wilkinson et al. 2003). These bacterial pathogens undoubtedly worsen a patient’s health over a long period of time and may be a major driver of airway luminal inflammation (Marin et al. 2012). For example, COPD patients chronically colonized with *H. influenzae* during stable phase also showed increased airway inflammation and reduced lung volumes when compared with non-chronically colonized patients (Tufvesson, Bjermer & Ekberg 2015).

The persistence of viruses in the airway has also been reported in the stable clinical phase of COPD, most commonly Respiratory Syncytial Virus (RSV), which has been detected in up to 30% of patients in some reports (Borg et al. 2003; Seemungal et al. 2001), but not others (Falsey et al. 2006). Thus, the role of RSV in COPD is still debated. The presence of the adenovirus hexon gene (as an index of presence of acute virus) has also been reported during both the stable phase in 6% of disease and during acute exacerbations in 7%. The presence of adenovirus 5 E1A (viral gene product, index of viral multiplication) in lung cells isolated from sputum samples in COPD patients occurred infrequently (~6%) (McManus et al. 2007); however, individuals positive for E1A had a lower FEV₁ compared with E1A negative individuals (Kasuga et al. 2009).

2.2.2 Infection in acute exacerbations of COPD (AECOPD)

The risk of mortality and morbidity from COPD escalates when frequent exacerbations intervene, usually as the general severity of the COPD

worsens, and both are likely driven by infection (Miravittles et al. 1999; Rosell et al. 2005; Sapey & Stockley 2006). COPD exacerbations are really nasty experiences for the individual patient, and the recovery is frequently slow, usually over weeks at least (Soler-Cataluna et al. 2005). Frequent exacerbations are also directly linked with further accelerated decline in lung function, increased local and systemic inflammation and decreased quality of life (Donaldson, GC et al. 2002; Osman et al. 1997; Papi et al. 2006; Patel et al. 2002). Thus, a vicious cycle is established once this phase of disease is reached (Figure 2.2) (Sethi & Murphy 2001).

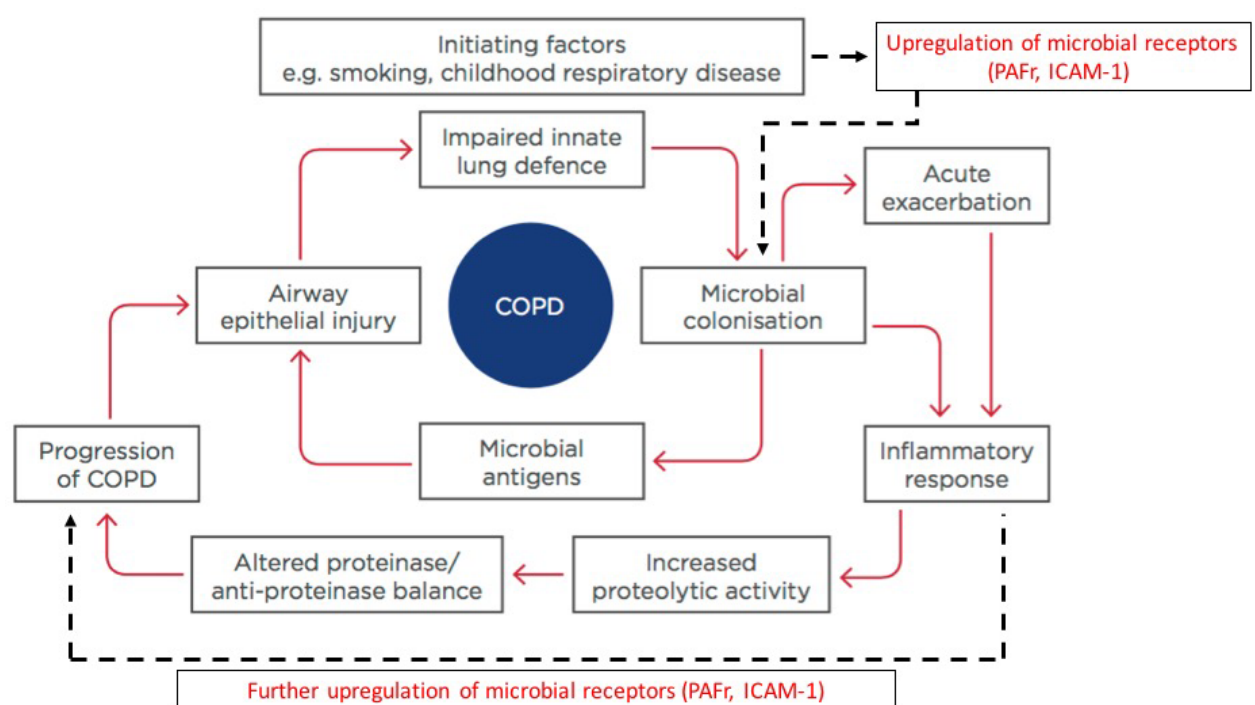


Figure 2. 2. The vicious-circle hypothesis of infection and inflammation in COPD.

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Both bacterial and viral pathogens have been implicated in AECOPD. A number of studies have reported that viruses are responsible for 40-60% of these episodes, with rhinovirus (RV) playing the major role (Mohan et al. 2010). RV has been described as triggering approximately 60% of all virus-

related exacerbations and 25% of all exacerbations (Hershenson 2013 George et al. 2014).

In addition to viral agents, bacteria have been implicated in up to 50% of exacerbations (Fagon et al. 1990), which is even higher (72%) in severe exacerbations requiring ventilatory support (Soler et al. 1998). Moreover approximately 25% of exacerbations of increased severity yielded bacterial-viral co-infection (Papi et al. 2006). Acquisition of new bacterial species, increase in the pre-existing bacterial load and acquisition of a new strain of established colonizing bacteria are thought to be the key factors implicated in bacteria-triggered AECOPD (Sethi et al. 2002; Sethi et al. 2004). Again, *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* are the most common bacterial pathogens involved in an AECOPD. Patients with AECOPD and *P. aeruginosa* present higher severity of COPD, while those with *S. pneumoniae* present greater comorbidity (Boixeda et al. 2015). Even in virally induced exacerbations, subsequent bacterial infection within a few days is a common clinical experience (experimentally induced HRV infections, Mallia et al. 2012; naturally occurring HRV infections, George et al. 2014).

A large USA-wide study (n=23,971) and reported that both current (light) smoking status and duration of smoking abstinence significantly reduced the risk of AECOPD (Au et al. 2009). Smoking cessation was also found to be associated with a reduced risk of hospitalization and total mortality in COPD patients (Anthonisen et al. 1994). Smoking history (pack-years) has been shown to be related to increased levels of lung/systemic inflammatory markers that could predict the risk of AECOPD, and inflammatory markers, including circulating C-reactive protein and TNF- α (Korani et al. 2016), soluble tumor necrosis factor receptor (sTNFR)-1 (Serapinas et al. 2011), and immune cells (predominantly macrophages in bronchial biopsies) (Willemse et al. 2005) have all been shown to be increased in COPD patients. However, the examiner is correct in that a strict relationship between smoking status and/or smoking history (pack-years) and exacerbation frequency has not always been observed (Cao et al. 2006; Donaldson et al. 2013; Gompertz et al. 2001; Han et al. 2013). One possible explanation for this lack of consistency is that patients experiencing frequent exacerbations are more

likely to quit, and smoking cessation certainly aids in improving COPD prognosis, slows lung function decline and improves the quality of life (Criner et al. 2015). It could be, though, that differences between individuals reflect the virulence of airway bacteria that they chronically carry, and some of this may depend on the details of ChoP expression, in terms of amount and position in the bacterial wall, but also the ease with which rhinovirus is able to infect the epithelium. Further work on this is now needed.

2.3 Mechanisms of chronic bacterial infection or ‘colonization’: general principles applicable to the respiratory tract

As discussed in earlier sections, a number of mechanisms could contribute to frequent risk of respiratory tract infections (both bacterial and viral) in COPD patients throughout the clinical course. This includes impaired host defense, compromised airway epithelial integrity and increased microbial adhesion receptors in the susceptible host. I will be focusing mainly on two microbial receptors, which could be very relevant to smokers and patients with COPD. Firstly, platelet-activating factor receptor (PAFr), a major attachment site for important bacterial pathogens, including NTHi, *S. pneumoniae* and *Pseudomonas aeruginosa*. Secondly, intercellular adhesion molecule-1 (ICAM-1), which is an important binding site for majority of human rhinoviruses (HRV-A and HRV-B) as well as NTHi.

Theoretically, the first important step in the initiation of pathogenic infective disease is adherence of the pathogen to a host surface, followed by subsequent invasion and clinical manifestations (Kline et al. 2009). Epithelial adhesion is essential for colonization of mucous membranes, for example on large and small airway epithelium in chronic airway disease, and presumably also the alveolar epithelial surface as a prelude to pneumonia (Pizarro-Cerda & Cossart 2006).

There is unlikely to be a sole “gold standard” mechanism of microbial colonization and invasion in host cells in the respiratory tract, reflecting the complex, dynamic and probably multifaceted nature of host-microbe

interactions. However, one relatively promising and potentially central approach relates to Platelet Activating Factor receptors (PAFr) (Grigg 2012). Notably, the two major respiratory pathogens in COPD, *H. influenzae* and *S. pneumoniae*, adhere to epithelial surfaces by physico-chemical interactions between phosphorylcholine $[(CH_3)_3N^+CH_2CH_2PO_4^-]$ or ChoP (a small zwitterionic/bipolar molecule and molecular mimic of PAF) on the bacterial cell wall surface with PAFr expressible on the luminal surface of respiratory tract epithelial cells (Cundell et al. 1995; Swords et al. 2000).

The interaction between this bacterial wall ligand (ChoP) and epithelial surface PAFr seems to be quite specific as only pathogens expressing ChoP are able to “anchor” to PAFr. Indeed, there is now a growing body of evidence from both in vivo and in vitro studies that PAFr is an important host epithelial receptor used by specific respiratory bacterial pathogens to colonize and then invade respiratory epithelium and deeper tissue (Clementi & Murphy 2011; Iovino et al. 2013). In theory, therefore, any factor that upregulates either optimal ChoP presentation on bacterial surfaces or PAFr expression on epithelial surfaces could be very important in disease pathogenesis. I will now summarize the current evidence for this mechanism being relevant in COPD.

2.3.1 Platelet Activating Factor receptor (PAFr)

PAFr is a member of the G-protein coupled receptor (GPCR) superfamily, which is characterized by seven trans-membrane domains can be expressed on the surface of a range of cell types, including the majority of cells of the innate immune system (i.e., macrophages, polymorphonuclear leucocytes, mast cells etc.) but also potentially at the apical (luminal) surfaces of bronchial epithelial cells (Shukla 1992). The gene encoding PAFr in humans (PTAFR) has been mapped on chromosome 1. Like most GPCRs, PAFr becomes coupled as hetero-trimeric structures comprised of α , β and γ subunits on the cell plasma membrane (Izumi & Shimizu 1995).

Physiologically, PAFr binds specifically to its natural ligand, platelet-activating factor (PAF) (Shukla 1992). PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-

phosphocholine, is a potent phospholipid 'mediator' of inflammation synthesized by various cells, including polymorphonuclear leucocytes (PMNs), macrophages, monocytes, endothelial cells, mast cells and platelets, which in turn can all carry surface PAF receptors (Nakamura et al. 1991). PAF is believed to be involved in numerous leucocyte functions, platelet aggregation and inflammation. Coupling of PAF to PAFr activates GTPase, triggering phospholipid turnover via phospholipases C, D and A₂ pathways, which feed the various lipid mediator pathway (e.g. prostanoids and leukotienes), and thereby also produce more PAF. It also activates protein kinase C and tyrosine kinases (Iovino et al. 2013). PAFr plays a vital role in inflammatory processes, which can be blocked by specific PAFr antagonists (Chen et al. 2008).

Localization of PAFr has been shown in lungs, brain, as well as platelets, endothelium and leucocytes. Previous publications have affirmed the role of PAFr in trafficking of leucocytes to remove inhaled respiratory pathogens (Cabellos et al. 1992) (Figure 2.3). Thus, PAF-PAFr binding may initiate a pro-inflammatory cascade to eliminate inhaled bacteria (Cabellos et al. 1992). Other significant functions of PAF include upregulation of adhesion receptors in the host cells as well as epithelium. A time-dependent increase of ICAM-1 expression on human eosinophils is also induced by PAF (Sagara et al. 1996). PAF has also been reported to upregulate TLR-4 in intestinal epithelial cells, which again augments inflammation (Soliman et al. 2010). Whether PAF itself upregulates PAFr in lungs has not been investigated, but it remains a possibility, which needs further investigation; indeed little is known about PAF respiratory functionality.

A wide variety of receptors, including platelet-activating factor receptor, present on the surface of macrophages can recognize bacterial pathogens (Mosser, DM 1994). However, the persistence of microorganism (especially *H. influenzae*), both extracellular (in biofilms) and intracellular (within respiratory epithelial cells and macrophages) has been reported (Forsgren et al. 1994; St Geme & Falkow 1990). This is attributed to immunological impairments of alveolar macrophages (AM), including diminished inflammatory cytokine responsiveness and phagocytosis of NTHi (Berenson,

Garlipp, et al. 2006; Berenson, Wrona, et al. 2006). Defective phagocytosis by macrophages was found to be significantly greater for NTHi than other respiratory pathogens, including *S. pneumoniae* and *Moraxella catarrhalis*, in COPD (Berenson et al. 2013). However, the relevance of bacterial binding via ChoP to PAFr expressed on the surface of inflammatory cells is not fully understood, but certainly raises the possibility that evasion of effective clearance could be enhanced, i.e. an evolved way for certain bacteria to avoid other mechanisms of destruction, while at the same time enhancing the inflammatory response (Figure 2). The interaction between host PAFr and bacterial ChoP has been summarized and reviewed (Clark & Weiser 2013; Thornton, Durick-Eder & Tuomanen 2010), though not primarily in the context of lungs or COPD.

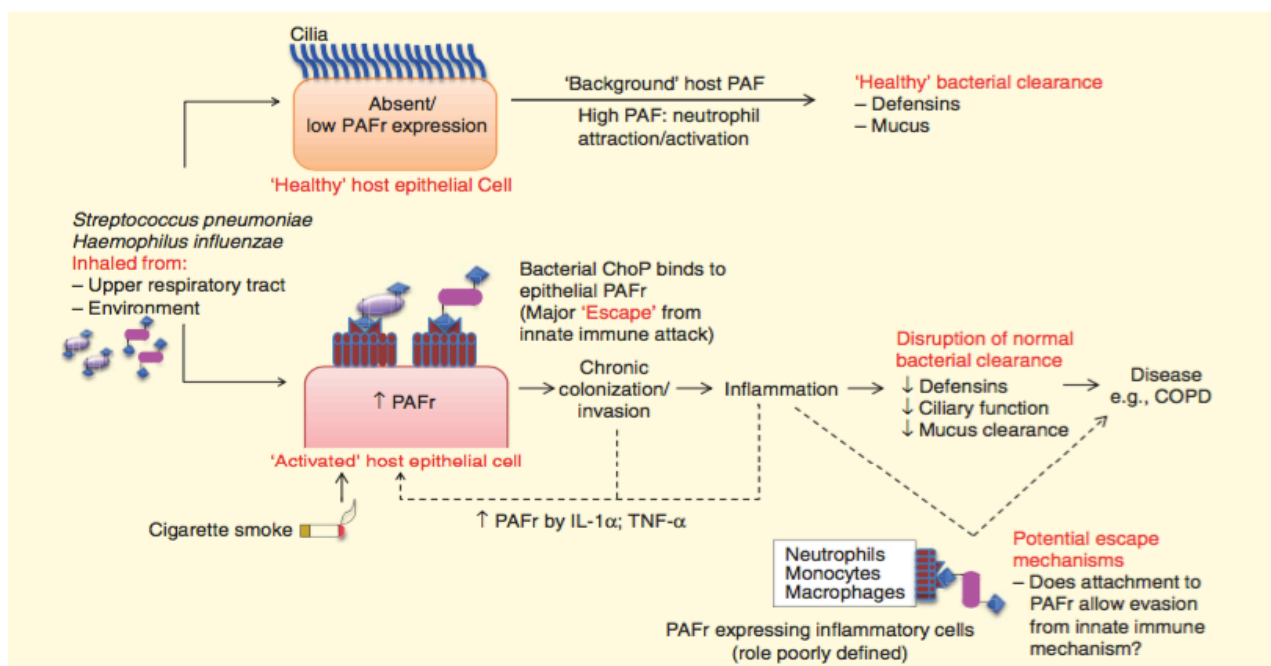


Figure 2. 3. Potential role of PAFr in clearance of inhaled bacteria.

PAFr probably plays a vital role in clearance of inhaled bacteria via interaction with PAF in healthy individuals. However, activated epithelium (e.g., cigarette smoke) with upregulated PAFr provides bacteria a possible escape mechanism from innate immune cells leading to chronic colonization, persistent inflammation and disease. For example in COPD, PAFr-expressing innate immune cells (macrophages) may also provide an escape mechanism especially for *Haemophilus influenzae* and lead to its persistence in the host.

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The first potential link between PAFr on lung structural cells and respiratory infections was provided by Cundell et al. (Cundell et al. 1995). They showed an interaction between *S. pneumoniae* and upregulated PAFr on cytokine-activated respiratory epithelial A549 cells as well as human umbilical vein endothelial cells (HUVEC), which was blocked by a PAFr antagonist (L659989). They also showed that virulent strains of *S. pneumoniae* adhered to PAFr more than non-virulent strains and reported a decrease of approximately 50% in adherence and internalization of *S. pneumoniae* into cells with the PAFr antagonist.

A strategic importance for PAFr expression specifically in airway colonization by the major respiratory pathogens *S. pneumoniae* and NTHi or in invasive pneumococcal disease has been previously suggested in both cultured cells and animal models (Iovino et al. 2013; Van Eldere et al. 2014). Further efforts to understand the interactive host-response mechanisms involved in airway respiratory infections in COPD are of vital importance. Upregulation of this PAF-PAFr system in other situations of chronic pulmonary infections, e.g., cystic fibrosis, or in response to environmental dust and fume exposure also needs to be investigated. Variation in both ChoP expression on bacteria and PAFr expression on epithelial cells, as well as their variable interactions could be important in the natural history of these diseases.

2.3.1.1 Mechanistic *in vitro* studies; importance of PAFr in invasive pneumococcal disease

In an animal “meningitis” model, Radin et al. reported co-localization of *S. pneumoniae* and PAFr in 82% of rat brain microvascular endothelial cells (BMEC) based on confocal microscopic analyses. A further noteworthy finding was increased expression of PAFr on endothelial cells in infection compared to non-infected animals. This suggests that *S. pneumoniae* may itself cause an upregulation of receptor expression once cells are activated and infection

has commenced (Radin et al. 2005). Whether PAF could be the intermediate mediator needs investigating.

Going a step further, Ring et al. showed *S. pneumoniae* 'navigating' eukaryotic cell layers (human and rat) of brain microvascular endothelial cells via transcytosis (a process for transportation of macromolecules through cells in vesicles, which is mediated by highly specific signal cascades). This process is believed to be an essential mechanism in the development of invasive pneumococcal disease. They reported a decrease of 35% in the amount of intracellular bacteria on treating the cells with a PAFr antagonist (L659989) prior to infection accompanied by a 25%-30% decrease in transcytosed bacteria (Ring, Weiser & Tuomanen 1998).

Although transcytosis as a process is well established, its exact mechanism is not entirely understood, and it needs more extensive investigation, perhaps especially in the human respiratory tract. However, the two major membrane receptors proposed to interact directly with pathogens are PAFr and polymeric immunoglobulin receptor (pIgR) (Iovino, Molema & Bijlsma 2014; Ring, Weiser & Tuomanen 1998), another putative bacterial receptor.

Indeed, inflammatory cytokines have also been shown to upregulate the epithelial expression of cytokeratin 10, pIgR and laminin receptor, which have all been shown to enhance adherence of *S. pneumoniae* to human cells (Shivshankar et al. 2011). Therefore, it could be that activation of human cells (epithelial and endothelial) by inflammatory cytokines induces upregulation of several receptors; that all play some role in elevated bacterial adhesion and subsequent cellular uptake of Pneumococci (Chao & Olson 1993). However, I will initially focus on the role of PAFr in COPD, since it is still likely to be the most important respiratory pathogen adhesin.

2.3.1.2 Importance of PAFr in non-typeable *H. influenzae* (NTHi) colonization and invasion

NTHi is a common commensal residing in the human nasopharynx, but it can cause opportunistic lower respiratory tract infection whenever an individual is compromised by either other infections or disease (Clementi & Murphy 2011). The lower respiratory tract connects with the upper respiratory tract at the “guardian” vocal cords, but frequent micro-aspiration of secretions takes place normally (Gleeson, Eggli & Maxwell 1997). Further, it remains a possibility that NTHi is normally inhaled from the upper respiratory tract to the lower respiratory tract but is effectively cleared in health by normal host defenses (Murphy 2003). However, in the case of smokers and COPD patients, this clearance does not occur so efficiently probably for a number of reasons, but the relative importance of NTHi epithelial cell adherence leading to invasion may be especially important, but not yet well worked up (Sethi & Murphy 2008). Bandi et al. reported the presence of intracellular NTHi in bronchial biopsies from 33% of stable chronic bronchitis patients, 87% of acutely ill chronic bronchitis patients but in no healthy tissue (Bandi et al. 2001). As already emphasized, NTHi is also an important cause of clinical acute exacerbations in COPD, and also in cystic fibrosis (Weinreich & Korsgaard 2008).

A limited number of studies of classical receptor-mediated adhesion and internalization of NTHi have already provided some valuable insights about the potential role of PAFr epithelial colonization and invasion by this bacterium (Swords et al. 2000; Weiser, Shchepetov & Chong 1997). Similar to *S. pneumoniae*, NTHi adhesion to PAFr is facilitated by phosphorylcholine (ChoP) precisely positioned enzymatically in the outer lipo-polysaccharide layer of the bacterium (Clark et al. 2012; Schweda et al. 2000; Weiser et al. 1998; Weiser, Shchepetov & Chong 1997). Variations in ChoP expression lead to variants of NTHi with modified virulence and function, such as variation in potency of bacterial LPS as an endotoxin, NTHi adherence, bacterial invasion, biofilm maturation, resistance to host antimicrobial peptides (such as cathelicidin LL-37) and resistance to pulmonary muco-ciliary clearance (Swords et al. 2000).

H. influenzae expresses ChoP ligand in the outer LPS layer (Schweda et al. 2000). The variation in virulence of NTHi is enzymatically controlled through both the amount of ChoP and its precise localization on the outer LPS. Both of these variables are controlled by moduable expression of genes in *lic 1* locus (McCrea, KW et al. 2010). There are four open reading frames, namely, *lic A* (choline kinase), *lic B* (choline permease), *lic C* (pyrophosphorylase) and *lic D* (diphosphonucleoside choline transferase) (figure 2.4) (Weiser, Love & Moxon 1989). *Lic A* and *lic B* gene are believed to regulate the amount of choline incorporated from the outer medium (host environment) and the biosynthesis of ChoP inside the bacteria respectively (Michel et al. 2006). *Lic C* and *lic D* are involved in the variation in the ChoP presentation and precise localization in the LPS layer (Fan et al. 2003). In addition, there are multiple alleles of the ChoP transferase gene *lic D* in *H. influenzae* strains. These variables provide crucial ‘flexibility’ to NTHi to survive inside the host, either by increased adherence to host cells or potential survival inside PAFr-expressing innate inflammatory cells, e.g. macrophages (Craig et al. 2001).

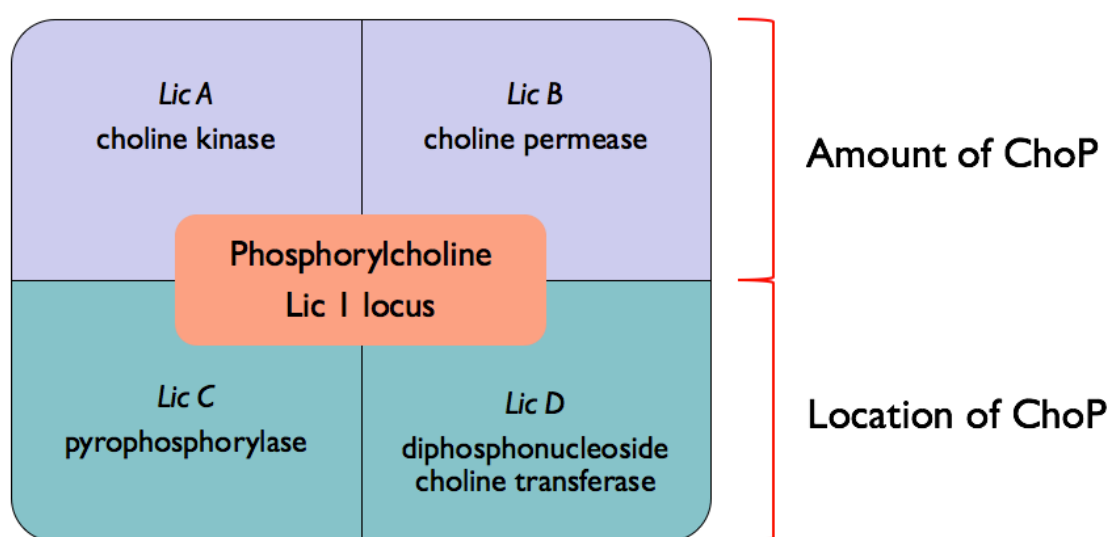


Figure 2. 4. Genetic control of phosphorylcholine in NTHi.

It is worth mentioning that in case of *S. pneumoniae*, ChoP is incorporated into cell wall C-polysaccharide (teichoic acid) and F-antigen (lipoteichoic acid) moieties (Fischer et al. 1993). However, little is known about the mechanisms controlling variation in either amount or localization of ChoP in *S. pneumoniae*.

Swords et al. concluded that ChoP–PAFr binding initiated host cell signaling through a PTX (pertussis toxin)-sensitive G protein complex that was required for optimal and efficient cell/tissue invasion. Thus ChoP-negative NTHi strains exhibited significantly lower invasion of bronchial epithelial cells, suggesting that ChoP–PAFr-mediated invasion is more efficient than other alternative mechanisms (Swords et al. 2001).

Human bronchial epithelial cells in culture infected by NTHi, likely using PAFr, showed a decreased bacterial load only after seven days from inoculation, implying that PAFr does aid in bacterial persistence in cells (Swords et al. 2000). Moreover, intra-epithelial bacteria are protected from the detrimental effects of antibiotics as well as bactericidal antibodies for at least 24 hours in vitro (Clementi & Murphy 2011). Interestingly the turnover of pulmonary epithelium is approximately 180 days, which could therefore potentially harbour bacteria safely for a protracted period (Rawlins & Hogan 2008). Little research in patient material has been done on this area and the clinical relevance of these findings remains unknown.

2.3.1.3 Mechanistic *in vivo* (mice) models of PAFr mediated lung infections

Two studies have assessed PAFr expression in aged mice, a model that might mimic COPD. Hinojosa *et al.* performed intranasal TNF- α challenge of BALB/c mice and reported an increase in PAFr expression in the lungs; they also found increased susceptibility of these animals to pneumococcal infection (Hinojosa, Boyd & Orihuela 2009). Likewise, Shivshankar *et al.* showed that aged mice (BALB/c), after intranasal challenge with *S. pneumoniae* had more lung inflammation as well as increased PAFr expression in the lungs (Shivshankar et al. 2011). It is interesting that more inflammation does not in these circumstances lead to better clearance of bacteria but indeed are a marker of bacterial escape from usual innate clearance mechanisms; this is again reminiscent of COPD in humans. Although murine models of COPD have provided us with some exciting insights regarding mechanisms involved in bacterial infections, these experiments may not be optimal because of

dissimilar inflammatory responses, and receptor pharmacology, and lack of the classic structural changes seen in human airways in COPD. More human work to back up suggestive animal findings has been urgently required.

2.3.1.4 Preliminary human tissue studies suggesting relevance of PAFr

The expression pattern of PAFr in both in vitro cell cultures and animal models have been reported, its relevance in COPD patients is not yet clear, and was the focus of my project. Moreover, the available studies only studied either *Pneumococcus* or NTHi in their experiments, which further compelled us to investigate both the bacterial pathogens in my project, given all the other experimental conditions are kept alike.

Until now, relatively few studies have assessed the possible role of PAFr in chronic or acute airway and lung colonization/infection in COPD. Paradoxically, the first study by Shirasaki and colleagues using northern blot assay in lung tissue obtained from smokers and asthmatics concluded that PAFr mRNA was significantly upregulated only in asthmatic individuals but decreased in the lung tissues of smokers (although chronic infection is only clinical characteristic in smokers) (Shirasaki et al. 1994).

Human data also exist. Thus, tracheal epithelial cells exposed to acid showed upregulation of both PAFr mRNA and protein levels as well as increased adhesion of *S. pneumoniae* to cells. Moreover, pretreatment of acid-exposed cells to a PAFr antagonist (Y24180) resulted in an approximately 30-40% reduction in *S. pneumoniae* adherence to cells (Ishizuka et al. 2001).

Perhaps more relevant to human environmental exposures, human-derived A549 cells as well as human primary bronchial epithelial cells exposed to urban pollution particulates showed elevated levels of PAFr mRNA transcript levels and PAFr protein expression. This was accompanied by an approximately threefold increase in pneumococcal adhesion. This effect was again attenuated by a PAFr antagonist (WEB2086) (Mushtaq et al. 2011);

whether the drug mainly works through blocking PAFr up-regulation by PAF, the receptor itself, or both, needs to be fully worked out.

Conversely, McCullers and colleagues reported that PAFr is not required by *S. pneumoniae* to adhere to A549 cells in vitro. This opens the possibility of more emphasis being needed for PAFr-independent mechanisms for the adherence and invasion of epithelial cells by Pneumococcus (McCullers et al. 2008). In addition, in culture studies, which have suggested a direct role of PAFr in adhesion and invasion of human cells by *S. pneumoniae*, treatment of these activated cells with PAFr antagonist never blocks bacterium-PAFr interaction completely. Most studies have reported a maximum of 50% reduction in bacterial transcytosis with PAFr antagonists, which again indicate the likely relevance of PAFr-independent pathways for bacterial adherence and invasion i.e. virulence.

More recently, Grigg et al. (Grigg et al. 2012) investigated the role of PAFr in adhesion of *Streptococcus pneumonia* to A549 cells (alveolar epithelial cell line), BEAS2-B cells (bronchial epithelial cell line) and HBEpC cells (human primary bronchial epithelial cells), exposed to cigarette smoke extract. They also determined the specific role of PAFr in increased adhesion of pneumococcus to all these cell-lines by blocking PAFr with a specific receptor antagonist (CV-3988). Furthermore, they quantified PAFr transcript level by quantitative real-time PCR and determined PAFr protein expression by flow cytometry. This novel study also had clinical tissue analysis section provided by our group, assessing PAFr expression in upper airway epithelium in limited biopsies from active smokers by immunostaining.

These studies concluded that increased pneumococcal adhesion to cells exposed to cigarette smoke extract occurred as a result of increased PAFr expression, and PAFr antagonist attenuated pneumococcal adhesion to epithelial cells. Mice exposed to CSE also showed significant upregulation in lung PAFr mRNA transcripts. Perhaps of most interest was suggestion of at least a small increase in PAFr-expression in active smokers compared to

normal non-smoking individuals (Grigg et al. 2012), in whom PAFr expression was essentially absent.

Suri et al. (Suri et al. 2014) induced experimental respiratory tract rhinoviral infection (RV16, group A HRV) in normal controls, normal smokers and smokers with COPD. They reported a significant increase in PAFr mRNA expression in airway biopsies from smokers and smokers with COPD at baseline, but with no change after viral infection. In contrast, RV-14 (group B HRV) infection has been reported to stimulate *S. pneumoniae* adhesion to airway epithelial cells via upregulation of PAFr (Ishizuka et al. 2003), and RV-16 infection significantly increased expression of host cell adhesion molecules, including PAFr, both at the gene and protein levels, in nasal epithelial cells. In the later study, increase in bacterial adherence was also found (Wang, Kwon & Jang 2009).

2.3.1.5 PAFr as a potential therapeutic target for treatment of COPD exacerbations

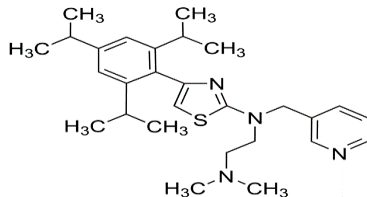
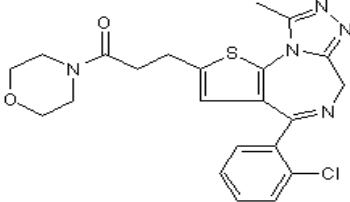
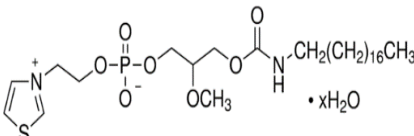
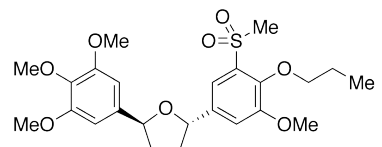
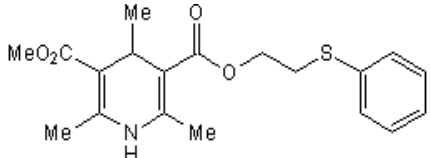
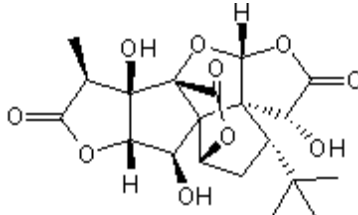
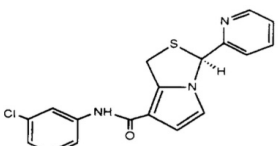
If one can summarize the evidence as suggesting that the major respiratory pathogens associated with COPD exacerbations, *S. pneumoniae* and *H. influenzae*, specifically bind to epithelial PAFr for colonisation of the lower respiratory tract, then PAFr represents a putative target for non-antibiotic therapies for both chronic COPD and especially exacerbated COPD (Bandi et al. 2001; Grigg et al. 2012; Ring, Weiser & Tuomanen 1998; Swords et al. 2000). As alluded to above, there are already several known antagonists of PAFr function, which could be categorized based on their chemical properties (Table 3).

In assays with rabbit platelet and rabbit polymorphonuclear leukocyte membranes, the antagonist, L659989 ((2R,5R)-2-(3-methoxy-5-methylsulfonyl-4-propoxyphenyl)-5-(3,4,5-trimethoxyphenyl)oxolane) competitively inhibited [³H]PAF binding with a low equilibrium inhibition constant (K_i) of 1.1 nM (Hwang et al. 1988). It was approximately 10-fold less active towards human platelet, human polymorphonuclear leukocyte and

human lung membranes, with a K_i of 14.3 nM indicating that it displayed a degree of specificity with regard to receptors from different hosts. L659989 was also a potent inhibitor of PAF-induced aggregation of rabbit platelets and degranulation of rat (50% effective dose (ED_{50}) of 4.5 nM) and human (ED_{50} 10 nM) neutrophils and was active following oral administration to female rats (ED_{50} 0.2 mg/kg) (Ponpipom et al. 1988).

In terms of effectiveness in humans, the PAFr antagonist CV-3988 ([2-methoxy-3-(octadecylcarbamoyloxy)propyl] 2-(1,3-thiazol-3-ium-3-yl)ethyl phosphate) in humans, in doses ranging from 750 to 2,000 μ g/kg, reduced platelet sensitivity for PAF (Arnout et al. 1988). In terms of drug tolerability, the drug did not cause any significant adverse events at these concentrations. However, clinically insignificant hemolysis was observed, possibly due to the detergent-like properties of the drug (Arnout et al. 1988). Serious adverse events were similarly not found with WEB 2086 (5mg-100mg daily), though small fluctuations of blood pressure and pulse were found in the treatment groups (Adamus et al. 1990).

Table 3. Known classes of PAFr-antagonists

Category	Name	Structure	Reference
1. Nitrogen heterocyclic compound	SR27417		(Bastos da Silva et al. 2000; Herbert et al. 1993)
	WEB2086		(Dent et al. 1989; Dent et al. 1989)
2. PAF analogue	CV3988		(Terashita, Imura & Nishikawa 1985) (Arnout et al. 1988)
	L659989		(Hwang et al. 1988; Ponpipom et al. 1988)
3. Dihydropyridines	PCA4248		(Ortega et al. 1990) (Fernandez-Gallardo et al. 1990)
4. Natural medicines	BN52021		(Desquand et al. 1986)
5. Others	52770 RP		(Terashita et al. 1992)

Existing PAFr antagonists may therefore provide specific inhibitors of PAFr-bacterial interactions. Indeed, Cundell and colleagues demonstrated that the potent PAFr antagonist L659989 inhibited the adhesion of *S. pneumoniae* to cytokine-activated primary human umbilical vein endothelial cells *in vitro* (Cundell et al. 1995). This translated into the blocking by L659989 of *S. pneumoniae* adherence to IL-1-activated lung epithelium and progression to pneumonia in a rabbit model of infection (Cundell et al. 1995). Although most of the modern long acting bronchodilator and steroid inhaled medications now available for COPD treatment claim some effect in decreasing exacerbation rates, little is known about how they work in doing this. Follow-up work is now needed to examine the capacity of PAFr antagonists and their structural analogues to selectively target the use of PAFr as a gateway for infection of the lower respiratory tract of COPD patients by *H. influenzae* and *S. pneumoniae*.

Furthermore, and as explained earlier, most exacerbations involve microbial infection (Bandi et al. 2003; Sapey & Stockley 2006; Sethi 2004), so whether these agents may affect host PAFr expression, bacterial ChoP expression, or the interaction between them, need to be investigated. In summary, all living organisms, including bacterial respiratory pathogens, have evolved strategies to survive within their specific niches. Inflammatory mediators, including PAF with its likely auto up-regulation of its own receptor, seem to be key pro-inflammatory components involved in countering foreign bacterial invasion. However, several pathogens, especially *S. pneumoniae* and *H. influenzae* have evolved mechanisms to specifically adhere to PAFr and use this to avoid innate immune attack, and also to potentially colonize the airway epithelial surface. In normal airway epithelium, we believe that PAFr expression is essentially absent, but is enhanced by cigarette smoking. Further work in COPD was needed and provided in this thesis.

2.3.2 Intercellular adhesion molecule-1 (ICAM-1)

Human ICAM-1 (CD54; a transmembrane glycoprotein) is a member of the immunoglobulin (Ig) superfamily that contains five Ig-like domains, a

transmembrane domain, and a short cytoplasmic tail (Springer 1990); it is thought to be expressed constitutively on a wide variety of cells (including leukocytes, endothelial cells and respiratory epithelial cells) (Roebuck & Finnegan 1999), but it is also further inducible by the inflammatory mediators TNF- α , IL-1 β and IFN- γ , and inhibited by glucocorticoids (Hubbard & Rothlein 2000). Membrane-bound integrin receptors such as, LFA-1 and Mac-1 on leukocytes, CD43, soluble fibrinogen and hyaluronan, matrix factor hyaluronan, rhinoviruses, and *Plasmodium falciparum* malaria-infected erythrocytes serve as ligands for ICAM-1.

Physiologically, ICAM-1 plays a number of key roles. It stabilizes cell-cell interactions and it also facilitates leukocyte per-endothelial transmigration from blood into inflamed tissues possibly leading to initiation of antigen-specific immune responses (Lehmann et al. 2003; van de Stolpe & van der Saag 1996). ICAM-1 functions in the T-cell-mediated host defense system, and also as a co-stimulatory molecule on antigen-presenting cells to activate MHC class II restricted T-cells, and on other cell types in association with MHC class I to activate cytotoxic T-cells.

The regulation of ICAM-1 at transcriptional level has been reviewed (van de Stolpe & van der Saag 1996), and It has been suggested that the expression of ICAM-1 is transcriptionally regulated through one of four pathways: NF- κ B, gamma interferon–Janus kinase/signal transduction-activating transcription (JAK/STAT), mitogen-activated protein (MAP) kinase/activator protein 1 (AP1), and, indirectly, protein kinase C (PKC) (Roebuck & Finnegan 1999). The ICAM-1 promoter contains several enhancer elements, but notably nuclear factor-kappa B (NF- κ B), which mediates effects of 12-O-tetradecanoylphorbol-13-acetate (TPA), IL-1, lipopolysaccharide (LPS), TNF- α , and glucocorticoids. Because of this complexity, the regulation of ICAM-1 expression is cell specific and depends upon the availability of cytokine/hormone receptors, signal transduction pathways, transcription factors, and post-transcriptional modification (Jahnke et al. 1995). Moreover, ICAM-1 is subsequently shed by cells and is detected in plasma as soluble (s)ICAM-1, and is found to be increased in many pathological conditions

including malignancies (e.g., melanoma and lymphomas), many inflammatory disorders (e.g., asthma and autoimmune disorders), atherosclerosis, ischemia, certain neurological disorders, and allogeneic organ transplantation (van de Stolpe & van der Saag 1996).

In addition to all of the above, there is strong evidence that ICAM-1 serves as an important receptor for major group of human rhinoviruses (HRV) (Greve et al. 1989), and for some coxsackie viruses (Marlin et al. 1990). For my studies, I focused on the potential role of cigarette smoke on the expression pattern of ICAM-1, which could potentially facilitate the adhesion and pathogenesis of HRV to host epithelial cells.

2.3.2.1 Human Rhinovirus

Human rhinoviruses (HRV), first isolated in the 1950s from individuals with common cold symptoms, represent one of the most serologically diverse groups of pathogenic picornaviruses (over 160 serotypes have already been identified) (Bochkov & Gern 2016). HRVs are classified into three species on the basis of phylogenetic relationships and sequence divergence: HRV-A, HRV-B, and HRV-C (Kuroda et al. 2015; McIntyre et al. 2013).

HRVs are positive-sense, single-stranded-RNA (ssRNA) viruses of approximately 7,200 base pairs. The viral genome consists of a single gene whose translated protein is cleaved by virally encoded proteases to produce 11 proteins. Four proteins, VP1, VP2, VP3, and VP4, make up the viral capsid that encases the RNA genome, while the remaining nonstructural proteins are involved in viral genome replication and assembly. The VP1, VP2, and VP3 proteins account for the virus' antigenic diversity, while VP4 anchors the RNA core to the capsid. There are 60 copies each of the four capsid proteins, giving the virion an icosahedral structure, with a canyon in VP1 that serves as the site of attachment to cell surface receptors.

As noted earlier, more than 90% of known HRV serotypes, the “major group,” utilize the cell surface receptor ICAM-1, while the “minor group” attaches to

and enters cells via the low-density lipoprotein receptor (LDLR). Some of the major-group HRVs also use heparan sulfate as an additional receptor (Palmenberg, Rathe & Liggett 2010). In short, HRV-A and -B bind to ICAM-1 on respiratory epithelial cells while HRV-C uses cadherin-related family member 3 (CDHR3) to accomplish cellular entry (Bochkov et al. 2015; Staunton et al. 1989)

2.3.2.2 Respiratory infections by HRV infection

HRVs chiefly cause upper respiratory tract (URT) infections, but may also infect the lower respiratory tract (LRT), in both children and adults (Hayden, FG 2004; Winther 2011). A number of studies using molecular techniques (RT-PCR) to detect HRVs have established respiratory viral infections as a major risk factor for acute exacerbations of asthma and COPD in both adults and children, frequently leading to hospital admission. HRV is also associated with wheezing illnesses in children, cardiopulmonary disease, and fatal pneumonia in immune-compromised patients. While other respiratory viruses, such as influenza virus and respiratory syncytial virus (RSV), cause a destruction of airway epithelial cells, HRV is not associated with destruction of the epithelial lining in nasal biopsies from subjects with natural 'colds' (n=29), determined by light or scanning electron microscopy (Winther et al. 1984).

Increased nasal ICAM-1 expression has been reported on the respiratory epithelium of allergic subjects (Gorska-Ciebiada et al. 2006). Malmstrom et al. observed that HRV was frequently found in the lower airways in infants (n=201; age 3-26 months) with recurrent respiratory symptoms. The presence of HRV was associated with increased resistance to airflow, which predicted subsequent asthma in later life (Malmstrom et al. 2006). HRV was detected by both immunohistochemistry and the indirect *in-situ* RT-PCR (n=20), in more bronchial biopsies of asthmatics (73%) than in non-asthmatic subjects (22%). Again, presence of HRV was associated with more airway obstruction, higher numbers of blood eosinophils and leukocytes, and eosinophilia in bronchial mucosa (Wos et al. 2008). Experimental HRV infection of human participants (n=19) reported the presence of virus in cells from nasal lavage (all subjects),

sputum (all subjects), bronchoalveolar lavage (26%), bronchial brushings (28%), and biopsy specimens (36%) (Mosser et al. 2005). Results from *in-vitro* primary cell culture models confirm that HRV infects lower airway tissues, and suggested viral replication in lower airway epithelial cells (Schroth et al. 1999), and a major role of ICAM-1 in the infective process (Terajima et al. 1997).

RNA-sequencing (RNA-seq) analysis of HRV-infected air-liquid interface (ALI) human airway epithelial cell cultures from 6 asthmatic and 6 non-asthmatic donors identified sets of host genes transcription associated with increased inflammatory pathways, epithelial structure and remodeling and cilium assembly and function (CCL5, CXCL10, CX3CL1, MUC5AC, CDHR3, CCRL1) (Bai et al. 2015).

As stated, HRV is an important trigger for acute exacerbations in COPD (AECOPD). Two prospective studies of patients hospitalized with AECOPD found that picornaviruses were the most common viral infection detected by RT-PCR in nasal lavage fluid, nasopharyngeal swab, or induced sputum specimens, occurring in 36 to 50% of cases (Kherad et al. 2010; Rohde et al. 2003). HRV prevalence and load at exacerbation was found to be significantly higher than in the stable state (53.3% versus 17.2%). COPD patients detected with HRV at exacerbation also had a higher exacerbation frequency per year compared with patients without HRV. However, secondary bacterial infection was common at about 14 days post-HRV infection (George et al. 2014), suggesting an interaction with PAFr regulation perhaps (Avadhanula, Rodriguez, Devincenzo, et al. 2006). Mallia et al. experimentally infected 13 COPD patients and 13 non-obstructed smoker controls with HRV and reported significant reductions in post-bronchodilator peak expiratory flow and carbon monoxide diffusion capacity from baseline to HRV infection in COPD patients but no change in controls. The authors also found increased neutrophil elastase and IL-8 levels in sputum supernatants and IL-6 levels in BAL in COPD patients infected with HRV. Although pulmonary AMs from BAL fluid have demonstrated deficient IFN- β responses in COPD patients compared to controls, there was only a non-significant trend toward reduced

levels of IFN- α and IFN- γ production. Interestingly, there was no change in TNF- α levels in either group (Mallia et al. 2011). Human bronchial epithelial cells exposed to airway secretions from subjects with bronchiectasis showed an increase in ICAM-1 mRNA and protein levels, in TNF- α -dependent manner (Chan et al. 2008).

Quint et al. examined serum IP-10 as a potential biomarker of HRV infection and reported that COPD patients (n=136) had higher serum IP-10 levels than age-matched controls without COPD (n=70), which also correlated with sputum HRV viral load. In the same study during 2-years of follow-up, the authors reported that serum IP-10 levels increased significantly from baseline during HRV-positive AECOPD and demonstrated no change during HRV-negative exacerbations (Quint et al. 2010).

Cigarette smoke exposure seems to be one of the most important risk factors for increased levels of cytokines and subsequent upregulation of ICAM-1, which could predispose smokers and COPD patients to frequent HRV-induced exacerbations.

2.3.2.3 Effect of cigarette smoke on ICAM-1

Small-airway epithelial cells from physiologically normal smokers (n=22) showed significantly higher levels of IL-8 and ICAM-1 mRNA than the non-smokers (n=17), suggesting that cigarette smoke is likely to increase epithelial ICAM-1 expression (Takizawa et al. 2000). Clinical studies have demonstrated elevated level of serum soluble (s)ICAM-1 in COPD-smokers (n=142) compared to non-COPD active smokers (n=55) (Lopez-Campos et al. 2012). An *in-vitro* study using primary human bronchial epithelial cells (HBEC) from COPD-smokers and non-smoking control participants reported a significantly increased release of sICAM-1 and IL-1 β in cells exposed to CS, compared to control air-exposed cells. In addition, exposure to CS also led to increased permeability in the cell culture layer from currently smoking participants (Rusznak et al. 2000). As stated in earlier sections, IL-1 β further

increases ICAM-1 expression on both the airway epithelial cells and endothelial cells.

HRV itself up-regulates membrane-bound ICAM-1 expression via a NF- κ B-dependent mechanism in both primary bronchial epithelial cells (12-fold) and the A549 respiratory epithelial cell line (3-fold) (Papi & Johnston 1999). Anti-ICAM-1 antibody (14C11), administered topically or systemically, has been shown to inhibit major group HRV replication *in vitro*, as well as HRV-induced inflammation, pro-inflammatory cytokine induction and lung virus RNA levels in a mice model (Traub et al. 2013), confirming a crucial role for ICAM-1 as a viral facilitator. Interestingly, anti-ICAM-1 antibody (14C11) did not prevent cell adhesion via human ICAM-1/LFA-1 interactions *in vitro*, suggesting the epitope targeted by 14C11 was specific for viral entry.

Although most attention has been on HRV, evidence indicates that ICAM-1 also serves as an adhesion molecule for *Haemophilus influenzae* (via bacterial P5 fimbriae), which is the main bacterial pathogen in COPD, again especially in AECOPD (Sethi & Murphy 2008). Thus, ICAM-1 is an attractive target to block not only for virus-receptor binding, but also to check ICAM-1-mediated NTHi adhesion to respiratory cells.

Despite the importance of ICAM-1 in exacerbations of COPD, its expression in the epithelium of COPD patients has not previously been directly investigated in large or small airways, which could be crucial for understanding the susceptibility for viral infections in the natural history of COPD. As mentioned earlier, approximately 90% of HRV bind specifically to ICAM-1 as their cellular receptor; so it is certainly is an attractive target for development of therapies.

2.3.3 Other receptors

The initial sensing of infection is mediated by innate, germline-encoded, pattern recognition receptors (PRRs) for sensing the presence of microorganisms, by recognizing structures conserved among microbial

species, which are called pathogen-associated molecular patterns (PAMPs). Recent evidence also indicates that PRRs are capable of recognizing endogenous molecules released from damaged cells, termed damage-associated molecular patterns (DAMPs). PRRs include transmembrane proteins, including toll-like receptors (TLRs), as well as cytoplasmic proteins such as RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) (Table 4). The specific roles of PRRs have been reviewed previously (Akira, Uematsu & Takeuchi 2006; Kumagai & Akira 2010; Takeuchi & Akira 2010).

TLRs are largely found on innate immune cells and structural cells. These receptors act through a common intracellular signaling pathway to increase the expression of inflammatory cytokines (TNF- α , IL-1, IL-12), chemokines (IL-8, MCP-1, RANTES), endothelial adhesion molecules (E-selectin), co-stimulatory molecules (CD80, CD86), and antiviral cytokines (IFN- α , IFN- β) (Chaudhuri et al. 2005; Hammad et al. 2009; Zuo et al. 2015). Several investigators have also reported reduced TLR expression in COPD on airway inflammatory cells, which would lead to limited pathogen identification and clearance, facilitating bacterial colonization, viral invasion and increasing the risk of exacerbations (von Scheele et al. 2011), especially when the PAFr/ICAM-1 are increased at the same time.

Novel studies suggest that the antiviral response of the host is dependent upon the recognition of a variety of viral components, especially genetic material, e.g., RNA by host-cytoplasmic proteinases released by the cytoplasmic helicase proteins RIG-I (retinoic-acid-inducible protein I) and MDA5 (melanoma-differentiation-associated gene 5). RIG-I is essential for the production of interferons in response to RNA viruses including rhinovirus, influenza, and RSV whereas MDA5 is critical for picornavirus detection. Additionally, genetically modified RIG-I^{-/-} and MDA5^{-/-} mice are highly susceptible to infection with all these RNA viruses compared to control mice (Kato et al. 2006). In a recent finding, severe COPD patients demonstrated a diminished cytokine response (granulocyte colony stimulating factor and IL-

1β) to PAMPs (Fan et al. 2016), which could potentially lead to impaired pathogen clearance from patients with advanced disease.

Table 4. Pattern recognition receptors (PRRs) and their ligands.

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PRRs	Localization	Ligand	Origin of the ligand
Toll-like receptors (TLR)			
TLR 1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR 2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR 3	Endolysosome	dsRNA	Virus
TLR 4	Plasma membrane	LPS	Bacteria, viruses, self
TLR 5	Plasma membrane	Flagellin	Bacteria
TLR 6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR 7 (Human TLR8)	Endolysosome	ssRNA	Bacteria, viruses, self
TLR 9	Endolysosome	CpG-DNA	Bacteria, viruses, parasites, self
TLR 10	Endolysosome	Unknown	Unknown
TLR 11	Plasma membrane	Profilin-like molecule	Protozoa
Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs)			
RIG-I (Retinoic acid-inducible gene)	Cytoplasm	Short dsRNA	RNA viruses, DNA virus
MDA5 (mela- noma differentiation-associated gene 5)	Cytoplasm	Long dsRNA	RNA viruses (Picornaviridae)
LGP2	Cytoplasm	Unknown	RNA viruses
NOD-like receptors (NLRs)			
NOD-1	Cytoplasm	Peptidoglycan (g-D-glutamyl-meso-diaminopimelic acid)	Bacteria
NOD-2	Cytoplasm	Peptidoglycan (muramyl dipeptide)	Bacteria

Investigators have reported that *Moraxella catarrhalis*, which binds to a basement membrane glycoprotein, namely laminin through 'Ubiquitous

surface proteins' (UspA1 and UspA2). The effect of chronic smoking on lower airway epithelial membrane integrity is well documented that exposes the basement membrane. Interestingly, the laminin layer in the basement membrane is significantly thicker in smokers than in non-smokers (Amin et al. 2003; Tan, Forsgren & Riesbeck 2006). We have focused on two microbial adhesion molecules, namely, PAFr (bacterial) and ICAM-1 (predominantly viral).

2.4 Current COPD therapeutics

The current treatment for COPD is a stepwise process, which firstly aims to alleviate symptoms, before additional pharmacological agents are used to target inflammation and reduce the severity and quantity of exacerbations. Therapeutic strategies in COPD need to be tailored to the patient's condition, and are guided by symptoms, disease manifestations and complications, especially the patient's risk of experiencing exacerbations (GOLD 2015). Both oral and inhaled medications have been used for patients with stable disease to reduce dyspnea and improve exercise tolerance, but overwhelmingly inhaled treatment is now dominant. Most of the medications used are directed at the following 4 potentially reversible causes of symptoms and airflow limitation in a disease state that has largely fixed obstruction:

- Bronchial smooth muscle contraction
- Bronchial mucosal congestion and edema/ mucus hypersecretion
- Airway inflammation
- Increased airway secretions

Treatment has often started with short-acting bronchodilators (e.g. beta2-agonist such as salbutamol) to relieve a patient's breathlessness, however these have largely been replaced by long-acting bronchodilators and now frequently a combination of long-acting beta agonist and long-acting beta-muscarinic, though the latter may be tried by itself first. In severe (Stage III) or very severe (Stage IV) COPD patients and/or in those with frequent

exacerbations, inhaled corticosteroids (ICS) are also prescribed and are typically administered as a combination inhaler with a long-acting beta₂-agonist bronchodilator (e.g. fluticasone propionate and salmeterol). Antibiotics are also widely prescribed by health professionals to treat exacerbations under the prevailing belief that most exacerbations are of bacterial origin, and to also take a cautious approach to prevent complications such as pneumonia (Ram et al. 2006; Vollenweider et al. 2012).

Oral corticosteroid is normally used for more severe AECOPD, but then the definition is rather subjective. Serious adverse health effects (especially with the use of ICS and antibiotics in COPD) have been associated with some of these therapeutic strategies making COPD treatment perhaps less early than it once seemed. For example, heart problems have been suggested with LAMAs and there is more definitive evidence of increased risk of pneumonia with ICS. Furthermore, the inappropriate stewardship and overuse of antibiotics is understood to be contributing to the rising issue of multi-resistant bacterial strains including virulent strains of *H. influenzae*, *M. catarrhalis*, and *P. aeruginosa*, and increased treatment costs associated with COPD (Kyd, McGrath & Krishnamurthy 2011; Pasquale et al. 2012).

2.4.1 Limitations with inhaled corticosteroid (ICS) therapy

The vast majority of COPD patients (~80%) use inhaled corticosteroids, as a single agent, combination inhaler with a LABA or both (Thomas et al. 2014), either alone or in combination with a long-acting β_2 agonist (Decramer, Janssens & Miravittles 2012). Clinical studies which have evaluated the effect of inhaled corticosteroids (ICS) on airway infections have been too small to detect important effect in COPD and somewhat conflicting data have been reported (Finney et al. 2014). More precisely, two studies using sputum cultures found no association between inhaled corticosteroid use and bacterial infection (Marin et al. 2012; Miravittles et al. 2010). Worryingly, another study based on quantitative PCR (qPCR) reported that higher doses of inhaled corticosteroids were actually associated with greater bacterial loads (Garcha et al. 2012).

Large clinical trials have suggested that the use of inhaled corticosteroids (ICS) increases the risk of pneumonia in COPD patients by two to three-fold (Calverley et al. 2007; Cates 2013; Crim et al. 2009; Kew & Seniukovich 2014). Findings from the TORCH study was the first to show significantly increased incidence of pneumonia in the ICS-treated groups, with the probability of pneumonia being 12.9% with placebo, 13.3% with long-acting β 2-agonist (LABA) monotherapy, 18.3% with inhaled corticosteroid monotherapy, and 19.6% in the inhaled corticosteroid in combination with LABA group. Although ICS treatment was found to be associated with an increased relative risk (RR) of pneumonia of 1.52 (95% CI 1.32–1.76), but there was no significant increase in pneumonia mortality (Crim et al. 2009). Similarly, the INSPIRE study compared fluticasone combined with salmeterol with the long-acting muscarinic antagonist tiotropium. They reported the probability of having pneumonia within 2 years as 9.4% on inhaled corticosteroids plus long-acting β 2-agonist group and 4.9% in the tiotropium-only group (Calverley et al. 2011).

One study reported impaired clearance of *Klebsiella pneumoniae* in mice due to fluticasone causing increased mortality (Patterson et al. 2012), and in a mouse model of allergic airway disease, budesonide also impaired host defense to *Pseudomonas aeruginosa* (Wang et al. 2013). Conversely, in other animal models and in in-vitro cell culture models, fluticasone reduced cellular adherence of *S. pneumoniae*, *H. influenzae*, and *P. aeruginosa* (Barbier, Agusti & Alberti 2008; Dowling et al. 1999). Use of inhaled corticosteroid in children with asthma was associated with increased pharyngeal carriage of *S. pneumoniae* (Zhang et al. 2013). In an observational study on COPD patients, prior use of ICS is reported to be independently associated with decreased risk of short-term mortality and use of mechanical ventilation after hospitalization for pneumonia (Cheng et al. 2014). Oral or systemic steroids are recommended for severe pneumonia in hospitals, and use of corticosteroids was associated with reductions in mortality (~3%), need for mechanical ventilation (~5%) and hospital stays (~1 day) (Siemieniuk et al. 2015).

2.4.2 Effects of inhaled corticosteroids on exacerbations

The major therapeutic aim in COPD is to prevent exacerbations. The use of ICS is widespread in patients with COPD due to its ability to reduce exacerbations. Several studies have concluded a beneficial effect of ICS in reducing the number of COPD exacerbations for patients, especially with advanced disease ($FEV_1 < 50\%$ predicted) (Calverley 2004). Other clinical trials strongly indicate that the use of ICS may reduce clinically relevant exacerbations by approximately 30% and improve health status of patients who have moderate to severe disease (Jen, Rennard & Sin 2012). Moreover, the withdrawal of ICS may likely result in increased risk of exacerbations and worsening of health status (Price et al. 2013).

A recent Cochrane review suggested marginal positive effect of LABA/ICS inhalers on exacerbation rates in COPD patients in comparison to those with LABA alone. Data were reviewed from nine studies, which together randomised 9921 participants. The rate ratio was 0.76 (95%CI: 0.68 to 0.84), which corresponds to one exacerbation per person per year on LABA and 0.76 exacerbations per person per year on ICS/ LABA (Ni et al. 2014).

2.4.3 Effects of inhaled corticosteroids on quality of life (QoL)

COPD exacerbations have a major impact on well-being and daily quality of life, which is usually measured by St. George's Respiratory Questionnaire (SGRQ) (Jones et al. 1992). A reduction in score of 4 is considered a clinically relevant subjective improvement. A worse score predicts a worse clinical outcome (Osman et al. 1997). Adding ICS to the pre-existing treatment has been shown to significantly improve QoL in patients with COPD, as well as some improvement in lung function and decrease in respiratory symptoms (Yildiz et al. 2004). However, there is limited capacity for reversibility of impaired lung function in COPD (Ceylan 2006).

2.4.4 Limitations with antibiotic therapy

Standard guidelines recommend antibiotic therapy for all three cardinal symptoms of COPD (chronic cough, dyspnea, increased sputum purulence and production), although there is no substantiated evidence to support this recommendation (GOLD 2015). There is strong evidence that long-term macrolide therapy may significantly reduce COPD exacerbation rates, but whether through an anti-inflammatory or antibiotic mechanism is not clear (Albert et al. 2011; Uzun et al. 2014). Short-term antibiotics are selected for the treatment of AECOPD episodes, according to local bacterial resistance patterns, although empirical therapy usually starts with the administration of a macrolide, tetracycline or an aminopenicillin with or without clavulanic acid, and all seem to work (or not) equally well.

Since there is such a large question mark over both the short- and long-term use of antibiotics in COPD, there is a growing demand for specific non-antibiotic treatments to prevent bacterial colonisation and AECOPD.

2.5 Concluding remarks for literature review and questions for the present thesis

There is obviously increasing interest in the role of microorganisms in the progression of COPD as well as their contribution to acute exacerbations. Indeed, the majority (70–80%) of exacerbations are recognized as related to infections. Notably, two respiratory bacterial pathogens (NTHi and *S. pneumoniae*) are isolated in a majority of COPD patients, both from stable stages of the disease and during the AECOPD. In addition, HRV have been implicated as a major etiological trigger in virus-induced exacerbations. Despite all the data, the mechanisms by which these pathogens gain access to the lower respiratory tract are poorly understood.

The first step of a pathogenic disease is the attachment of pathogen to host cells, leading to colonisation, multiplication and subsequent tissue invasion. Several specific questions arise regarding mechanisms: Firstly, whether cigarette smoking leads to an increase in adhesion sites or ‘receptors’ for microbes on the respiratory epithelium. Secondly, how importantly the

demographics (e.g. age, lung function, smoking history) of an individual may correlate with the microbial adhesion receptors in both smokers and COPD patients. Thirdly, how feasible is it to develop an in vitro epithelial cell culture model that would mimic the smoking-induced airway epithelial cell changes, and whether it is possible to measure the increase of these receptors, both at a molecular level and protein expression level. Fourthly, whether blocking microbial receptors would result in reduced pathogen adhesion, with a potential for therapeutic application.

Thus, the **aims** of my thesis were developed to answer some of the early events that could lead to lower respiratory epithelial colonization in smokers and COPD patients, and are summarized below;

1. To assess PAFr protein expression in epithelium of large airways using mucosal biopsies in smokers and COPD patients. In addition, I also evaluated the effect of inhaled corticosteroids on the expression of PAFr in stored biopsies from a previous intervention study.
2. To assess PAFr expression in the epithelium of small airways and lung parenchyma in smokers and COPD patients, as Small airway and especially lung parenchyma is more relevant to pneumonia development.
3. To assess the change in PAFr expression in cultured BEAS-2B cells exposed to CSE, and use this as a model to evaluate the adhesion pattern of *S. pneumoniae* and NTHi in relation to PAFr expression, and to assess the effect of a specific PAFr antagonist (WEB-2086) on bacterial adhesion to CSE pre-treated BEAS-2B cells.
4. To assess epithelial ICAM-1 expression on the epithelium of large and small airways, as well as lung parenchyma, in smokers and patients with COPD. I also evaluated the effect of CSE on ICAM-1 expression in the *in-vitro* epithelial cell culture model (i.e. BEAS-2B cells exposed to CSE).

2.5.1 Hypothesis

From my aims, it can be seen that my studies are focused on "host factors" facilitating the adhesion of major respiratory pathogens to lung epithelium, both in human tissue (*ex vivo*) acquired from Royal Hobart Hospital and in an *in vitro* epithelial cell culture model. The impetus for my interest was the preliminary findings from an international collaborative study (Grigg et al., 2012), in which our group provided preliminary evidence of an increase in airway epithelial expression of PAFr in smokers (in both humans and mice model). This encouraged me to test the hypothesis that the microbial adhesion molecules PAFr and ICAM-1 are upregulated in airway epithelium in current smokers and patients with COPD, and may be highly relevant to the progression of this disease.

Chapter 3

Materials and Methods

3.1 Introduction

For the *ex-vivo* study of epithelial expression of adhesins, I investigated current smokers with established COPD (COPD-smokers), ex-smokers with COPD (COPD-ex smokers), current smokers with normal spirometry (termed as 'normal lung function smokers', NLFS), and normal healthy, never-smoking controls (NC) by immunostaining for markers of microbial adhesion factors, in particular PAFr and ICAM-1. In a longitudinal analysis, I also used the material that had been collected in a double blind randomised controlled trial comparing fluticasone propionate (0.5 mg/twice daily) with placebo for 6 months (Reid et al. 2008), and assessed the effects of ICS on the epithelial expression of PAFr.

I used epithelial cell cultures of BEAS-2B cells to validate these immunohistochemical findings. Briefly, BEAS-2B cells were cultured and stimulated with cigarette smoke extract (CSE). The mRNA transcript levels as well as protein expression were quantified for both PAFr and ICAM-1. Bacterial adhesion was assessed in relation to PAFr staining and a specific PAFr-antagonist (WEB-2086) was analysed for its ability to block the bacterial adhesion to CSE-pretreated epithelial cells. Materials and methods for these studies that are presented in chapter 4, chapter 5, chapter 6 and chapter 7, are detailed in this chapter.

3.2 Subject recruitment

3.2.1 Ethics statements

The Tasmania Health & Medical Human Research Ethics Committee approved all studies (EC00337, H0013051 and H0012921). All subjects gave

written, informed consent to use their tissue, either prior to volunteer bronchoscopy and biopsy, or prior to lung surgery.

3.2.2 Endo-bronchial biopsies (large airways)

Seventeen current smokers with established COPD (COPD-CS), 16 current smokers with normal lung function (NS), 15 ex-smokers with COPD (COPD-ES) and 15 normal healthy, never-smoking controls (NC) were recruited by advertisement in local newspapers and placement of posters in clinic waiting areas in the Royal Hobart hospital, as well as on the notice boards of social and veterans clubs (Table 5). Potential participants were interviewed and examined by a respiratory physician clinically and physiologically. The diagnosis of COPD was made according to GOLD guidelines based on FEV₁/FVC ratio and patients categorized into two groups on the basis of current versus ex-smoking history.

For normal lung function current smokers the inclusion criteria were; a minimum 10 pack-year history of cigarette smoking with spirometry within normal limits, i.e., FEV₁ (forced expiratory volume in one second) >80% of predicted, and FEV₁/FVC (forced vital capacity) >70%) and no scalloping out of the expiratory descending limb of the flow-volume curve to suggest small airway dysfunction. Normal healthy volunteers had no history of respiratory illness or smoking and had normal lung function.

In the longitudinal study of inhaled corticosteroid, a computer generated random numbers table was used to assign COPD participants to fluticasone propionate (FP) (Accuhaler; Glaxo-Wellcome, Middlesex, UK) 500 µg/twice daily or placebo via identical multi-dose dry powder inhaler devices for six months (Figure 4.1). Before and after treatment, lung function and bronchial biopsies were obtained. Half of the participants were current smokers and other half ex-smokers. This trial was registered with the Australian New Zealand Clinical Trials Registry (Registry No. ACTRN12612001111864). The details of the RCT is published previously (Reid et al. 2008).

Table 5. Demographic and lung function data for participants in the cross-sectional study (bronchoscopy) and at baseline in the ICS intervention.

Groups (numbers)	NC (n=15)	NLFS (n=16)	COPD-smokers (n=17)	COPD- ex smokers (n=15)	ICS at baseline (n=22)
GOLD I/GOLD II‡	N/A	N/A	10/7	8/7	10/12
Male/female	7/8	12/4	9/8	9/6	8/14
Age (years)	44 (20-68)	50 (30-66) (p=0.313)	61 (46-78) (p=0.001)*	62 (53-69) (p=0.001)*	60(46-69)
Smoking (pack years)	0	32 (10-57)	45 (18-78)	51 (18-150)	42(18-150)
FEV ₁ % predicted (Post BD)†	113 (86-140)	99 (78-125) (p=0.01)*	83 (66-102) (p<0.001)*	83 (54-104) (p<0.001)*	77(55-112)
FEV ₁ /FVC % (Post BD)†	82 (71-88)	77 (70-96) (p=0.218)	59 (46-68) (p<0.001)*	57 (38-68) p<0.001)*	58(41-68)

Data expressed as median and range.

NC- Normal control; NLFS-Normal lung function smoker; COPD-CS-COPD current smoker; COPD-ES- COPD ex-smoker; ICS-COPD patients on inhaled corticosteroids; N/A-Not any

*Significance difference from NC

† Post BD values after 400µg of salbutamol

‡ Diagnosis of COPD was made according to GOLD guidelines (GOLD 2013).

3.2.2.1 Exclusion criteria

1. Subjects with a history suggestive of asthma, which included symptoms in childhood, related significant atopic disorders, eczema or hay fever, substantial day-to-day variability or prominent nocturnal symptoms, or a history of wheeze rather than progressive

breathlessness and any who had previously used ICS (oral or inhaled) were excluded.

2. Significant uncontrolled comorbidities such as diabetes, angina or cardiac failure, and other coexisting respiratory disorders including pulmonary fibrosis, lung cancer and bronchiectasis.
3. Subjects with inability to give written informed consent were also excluded.

3.2.2.2 Inclusion criteria

1. Current-smokers with COPD aged at least 40 years with smoking history equal to or more than 15 pack-years and subsequently obtained BAL fluid had to be free of bacterial pathogens; FEV₁ 40% to 80% predicted, with FER (FEV₁/FVC) ≤ 70% post bronchodilator with definite scalloping out of the descending limb of flow-volume loop on spirometry. COPD ex-smokers with at least six months of smoking cessation were included.
2. Normal healthy never smoking controls and current smokers with normal lung function recruited also underwent bronchoscopic examination and physiological evaluation. They were at least 18 years old and FEV₁/FVC ratio of 70% or higher and FEV₁% predicted of 80% or higher.
3. None of the never-smoking controls individuals had any history of significant respiratory illness or smoking. For normal lung function current smokers the inclusion criteria were; less than 10 pack-year history of cigarette smoking with spirometry within normal limits (FEV₁ (forced expiratory volume) > 80% of predicted, and FEV₁/FVC (forced vital capacity) > 70%) and no scalloping out of the expiratory descending limb of the flow-volume curve, suggesting small airway dysfunction.

3.2.3 Bronchoscopy

Bronchoscopy was performed using standard techniques. Briefly, subjects were pre-medicated with nebulized salbutamol (5 mg) 15–30 min before the procedure. Sedation was achieved with intravenous midazolam (3–10 mg) and fentanyl (25–100 µg). Lignocaine (4%) was used for topical anesthesia above the vocal cords and 2% lignocaine was used to anaesthetize the airways below the cords, in 2 ml aliquots as required, up to a maximum of 6 ml. Subjects were monitored by pulse oximetry throughout the procedure and oxygen was administered routinely. Eight biopsies from secondary carinae of segmental and sub-segmental bronchi in the right lower lobe were obtained. There were no complications from the procedures. Bronchial biopsies were fixed in 4% neutral buffered formalin for 2 hours and subsequently processed into paraffin through graded alcohol and xylene using a Leica ASP 200 tissue processor.

3.2.4 Resected lung tissue (small airways)

A total of thirty-six patients were included. All had primary non-small cell lung cancer (NSCLC), with an approximately equal distribution of squamous and adenocarcinoma. Patients were classified as current smokers or ex-smokers (at least 12 months of smoking cessation). Eighteen patients had demonstrated GOLD stage I/II COPD on post-bronchodilator spirometry ($FEV_1 < 70\%$), and ten patients had small airway disease (SAD) only, with scalloping of the expiratory limb of the flow-volume curve and $FEF_{25-75} < 68\%$ predicted. In addition, there were eight individuals who were current smokers with no evidence of airflow obstruction, and hence designated as smokers with normal lung function (NLFS). Again, those with a history of other chronic respiratory disorders were excluded (Table 6), including anyone with a history or clinical/physiological suggestion of asthma. Surgical resection material well away from the main tumour, and containing non-cancer affected small and large airways, were fixed in formalin within minutes of surgery. At processing, tissue blocks of small airway ($< 2\text{mm}$ internal diameter) were embedded in paraffin for our analyses. For the ICAM-1 expression, we merged the COPD groups with SAD group (table 7).

Sections from nine non-smoking, non-COPD subjects were included as a control group (NC) for comparison. We are thankful to Prof. Darryl Knight (University of Newcastle, Australia) and Prof. J.C. Hogg (University of British Columbia, Canada) for assistance in providing normal small airway tissues. Emeritus Professor HK Muller, a professional pathologist, inspected and confirmed that these tissues were normal, and without the hallmarks of smoking or asthma.

Table 6. Demographic and lung function data for participants in the cross-sectional study (resected lung tissue for PAFr).

Study groups	NC	NLFS	SAD	COPD smoker	COPD- ex smoker
n	9	8	10	8	10
Male/female	6/3	3/5	2/8	4/4	6/4
Age (years)	52 (42-63)	72 (52-79)	63.5 (42-84)	63 (59-78)	68.5 (56-85)
Smoking history	N/A	23 (0.3-60)	40 (0-72)	30 (2-50)	33 (18-36)
FEV ₁ /FVC (%)*	N/A	79.5 (70-90)	73.4 (69-76.7)	66.0 (59.9-70)	63.9 (54.9-69)
FEF _{25-75%} L/sec Post	N/A	81.5	47.0	37.0	40.5
BD %pred.		(70-116)	(30.8-69)	(28-47)	(20-55)

Data expressed as median and range.

NC, Normal control; NLFS, Normal lung function smoker; COPD-CS, COPD current smoker; COPD-ES, COPD ex-smoker; N/A, Not any; SAD, small airway group.

*Post BD values after 400µg of salbutamol

Table 7. Demographic and lung function data for participants where resected lung tissue was used in the ICAM-1 study.

Study groups		NC	NLFS	CAL
	LA biopsy	SA resected tissue		
n	9	9	8	29
Male/female	4/5	6/3	3/5	13/16
Age (years)	65 (52-72)	52 (42-63)	72 (52-79)	66 (42-85)
Smoking history	N/A	N/A	23 (0.3-60)	32.5 (0-72)
FEV ₁ /FVC (%)*	N/A	N/A	79.5 (70-90)	68 (54.9-78)
FEF _{25-75%} L/sec Post BD %pred.	N/A	N/A	81.5 (70-116)	41.5 (20-69)

Data expressed as median and range.

CAL, chronic airflow limitation; LA, large airways; NC, Normal control; NLFS, Normal lung function smoker; N/A, Not any; SA, small airways.

*Post BD values after 400µg of salbutamol

3.3 Processing of biopsies and resected tissue

Sections were cut at 3 µm intervals from individual paraffin blocks and stained with hematoxylin & eosin and morphologically assessed for quality and lack of damage. Following removal of paraffin and rehydration, immunostaining for PAFr and ICAM-1 was done.

3.3.1 General procedure for hematoxylin and eosin (H&E) stain

1. Paraffin blocks were sectioned at 3µms after cooling in –20 freezer or on ice blocks 5-10 minutes.
2. For biopsies, two sections (approximately 3µm thick) separated by 40-50 (approximately 10 sections) were cut and put on each slide [3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50°C)] or Dako FLEX IHC microscopic slides (Code K8020, Dako Denmark A/S). For resected tissue, only one section of tissue was used due to greater size.
3. Slides were dried on hotplate at 56°C for one hour and overnight at 37°C.
4. Slides with tissue sections were placed in Mayer's hematoxylin for 5 minutes to elaborate nuclei, followed by rinsing in running water.
5. Tissue sections were placed in approximately in 400ml of water with 8 drops of ammonia 30 seconds, and rinsed well in running water.
6. Slides were then placed in eosin solution for 2 minutes, and rinsed quickly in running water to remove excess eosin and then placed into 95% ethanol for 30 seconds with agitation.
7. Further dehydrated with 3 changes of 100% ethanol was carried out (1 minute each).
8. Clearing of sections was done using two changes of fresh xylene (2 minutes each).
9. Sections were mounted in Depex using Dako Coverslipper (Dako, Denmark A/S) and dried on hotplate overnight.

3.3.2 General procedure for PAFr and ICAM-1 staining

1. Paraffin blocks were sectioned at 3µms after cooling in –20 freezer or on ice blocks 5-10 minutes.
2. For biopsies, two sections (approximately 3µm thick) separated by 40-50 (approximately 10 sections) were cut and put on each slide [3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in

milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50°C]] or Dako FLEX IHC microscopic slides (Code K8020, Dako Denmark A/S). For resected tissue, only one section of tissue was used due to greater size.

3. Slides were dried on hotplate at 56°C for one hour and overnight at 37°C.
4. Slides were labelled using printed labels and marked on the edges with wax pen.
5. Dewaxing of sections was done in “xylene” (twice for 5 minutes each) in fume hood.
6. Hydration to water was carried out using 100% ethanol followed by 95% then 70% (3 minutes each) followed by rinsing the sections well in running tap water (2 minutes).
7. Antigen retrieval was performed in PT Link (Dako, Denmark A/S): for PAFr, sections were kept at 95°C at high pH buffer for 20 minutes: for ICAM-1, a temperature of 100°C at high pH buffer for 15 minutes was used.
8. After antigen retrieval, the slides were cooled down to room temperature and transferred to Dako Autostainer Plus (Dako, Denmark, A/S), in which a program template for staining was made.
9. For both PAFr and ICAM-1, sections were placed in 3% H₂O₂ in distilled water for 20 minutes and washed in distilled water for 2 minutes to block endogenous peroxidase activity. For ICAM-1 staining, the sections were also blocked with non-specific non-serum protein block (X0909, Dako, Denmark A/S) for 10 minutes.
10. Sections were rinsed in working Tris HCl buffer for 2 minutes after each treatment.
11. Primary antibodies were applied to the sections; PAFr (11A4, clone 21, Cayman Chemicals, Michigan, USA, Catalogue No 160600, 1/80 dilution for 1 hour) and ICAM-1 (Merck Millipore Corporation, Merck KGaA, Darmstadt, Germany, Catalogue No MAB2130, 1/250 dilution for 90 minutes at 20°C, post heat retrieval). A negative control was also used: for PAFr-isotype-matched immunoglobulin IgG2a (X0931, Dako, Denmark A/S). For ICAM-1- isotype-matched immunoglobulin IgG1

- (X0943, Dako, Denmark A/S). Lung sections from previous biobank were used as positive controls for confirmation of staining.
12. Sections were rinsed well using Tris HCl (thrice, 5 mins each).
 13. Apply EnVision+ system-HRP labelled polymer (catalogue number K4001; Dako, Denmark A/S) reagent to sections for 30 mins and rinsed with Tris HCl buffer.
 14. DAB+ (catalogue number K3468; Dako, Denmark A/S) was applied to sections for 10 minutes and rinsed well using Tris HCl (twice) followed by distilled water.
 15. The slides were taken out of Autostainer and put in Mayers haematoxylin to elaborate nuclei for 3-5 minutes, then rinsed in running water.
 16. Sections were placed in approximately 400 ml of water with 6-8 drops of ammonia for 30 seconds, and rinsed well in running water.
 17. Dehydration was carried out in 95% ethanol, then two changes of 100% ethanol (2 minutes each).
 18. Clearing was done in two changes of xylene (2 minutes each).
 19. Sections were mounted in Depex using Dako Coverslipper (Dako, Denmark A/S) and dried on hotplate overnight.

3.4 Biopsy section quantification

Computer-assisted image analysis was performed with a Leica DM 2500 microscope (Leica Microsystems, Germany), Spot Insight-12 (Spot Imaging Solutions, USA) digital camera and Image Pro Plus 7.0 (Media Cybernetics, USA) software. Firstly, as many pictures as possible were taken of the tissue from the area of interest (for this study it was mainly epithelium, Rbm, lamina propria, sub-mucosal glands and lung parenchyma), avoiding overlapping of tissue. All image analyses were done using the above described image analyser. Randomly selected images (five for EBBs and eight for resected tissue) from the total number of images were used for desired measurements.

3.4.1 Quantification of PAFr expression

Epithelial PAFr expression in large and small airways was expressed as the percentage of epithelium with PAFr expression. PAFr-expressing cells in airway reticular basement membrane (Rbm) and were quantified and normalized over length of Rbm. Alveolar epithelial PAFr positive cells (both type I and type II pneumocytes) were quantified and normalized over the length of parenchymal wall. We also observed macrophages staining positive for PAFr and an additional quantification of PAFr expressing inflammatory cells was done which was normalized over the length of alveolar wall.

3.4.2 Quantification of ICAM-1 expression

ICAM-1 expression in both the large and small airway epithelium was expressed as the percentage of ICAM-1-expressing cells out of the total cells. Moreover, ICAM-1 expression was differentiated between being basal or global. An additional quantification of ICAM-1 expression in the goblet cells and sub mucosal glands was done in large airways only, with staining intensity evaluated as: 0, negative; 1, weak; 2, moderate for <20% of cells; 3, moderate for >20% of cells; 4, strong for >20% of cells. ICAM-1-expressing cells in airway reticular basement membrane (Rbm) were also quantified and normalized over the length of Rbm. For alveolar ICAM-1 expression, the number of type I and type II pneumocytes expressing the antigen were quantified as percent of total alveolar epithelial cells. The length of alveolar wall quantitated was approximately 6,500 µm, which was equivalent to around 50 cells/mm of alveolar wall.

The cell distribution of staining was also noted qualitatively. All slides were coded and randomised by an independent person and then counted in a single batch by me blinded to subject and diagnosis, with quality assurance on randomly selected slides provided by a professional academic pathologist (Professor H.K. Muller).

3.5 In vitro epithelial cell culture

A primary immortalised cell line of bronchial epithelial cells, BEAS-2B (CellBank, Australia), which is commonly used as an *in vitro* model for studying bacterial infections was used in this study. BEAS-2B culture work was conducted using an Ultima Class II Fume Safety Cabinet (LAF technologies, Australia) under sterile conditions. Cells were maintained at 37°C, 5% CO₂ in bronchial epithelial cell growth medium (BEGM; Lonza, Switzerland) supplemented with a bullet kit containing bovine pituitary extract, epinephrine, gentamicin, human epithelial growth factor, hydrocortisone, insulin, retinoic acid, and transferrin provided by the manufacturer (Lonza, Switzerland). Sterile sealed 75-cm² cell culture flasks (T75 flask; Corning, USA) were incubated in 4 mL of collagen (Life Technologies, USA) and PBS at a 1:30 ratio for 45 minutes. The solution was then aspirated before the addition of cell-containing solution.

BEAS-2B cells were obtained from liquid nitrogen storage, thawed at 37°C and suspended in 4 mL of pre-warmed culture media. The cell suspension was centrifuged (Beckman Coulter, USA) at 1500 RPM for 5 minutes at room temperature to separate cells from the cryoprotectant diluent dimethyl sulfoxide (DMSO; Sigma Aldrich, USA).

Upon subculture, cells were incubated at 37°C, 5% CO₂ for 5 minutes in 2.5 mL Trypsin-EDTA solution (Sigma Aldrich, T3924, USA) supplemented with 0.5% Polyvinylpyrrolidone (PVP; Sigma Aldrich, PVP40, USA) and mechanically agitated to ensure detachment of cells. The cell suspension was transferred to sterile centrifuge tubes and neutralised via the addition of fresh culture media, equivalent to four times the volume of solution and centrifuged at 1500 RPM for 5 minutes. Following centrifugation, the supernatant was aspirated, with the cell pellet mixed in fresh culture media at a concentration of 1.6×10^5 cells per mL. Cells were then utilised seeded in fresh T75 flasks, sterile clear-flat bottom 24-well plates (Corning, USA), sterile Millicell EZ-Slide 8-well glass plates (EMD Millipore, USA) at a concentration of 1.6×10^5 cells/mL, or sterile clear bottom 96-well plates (Sigma Aldrich, USA). The chosen cell density was optimised to ensure a monolayer of cells was present in each assay. Culture media was aspirated and replenished every 2 days

before subculture at 80-90% confluence for each experiment. Cells from 18 passages or less were used for all experiments.

Cell density was quantified using an improved Neubauer haemocytometer (Hausser Scientific, USA). A 10 μ L aliquot of cell-containing solution was mixed thoroughly with Trypan Blue (Sigma Aldrich, USA) at a 1:1 concentration. 10 μ L of this solution was pipetted between a glass cover slip and haemocytometer via capillary action. The haemocytometer's grid was seen using an Olympus Light Microscope (Olympus; Tokyo, Japan) at 200x magnification. Four sections of the counting chamber were used to count cells, with each section having a total volume of 0.1 mm³ per section (1 μ L). The average cell count was multiplied by two to account for the dilution factor before being multiplied again by 10 000 to calculate the number of cells per mL. Only viable cells, which were unstained by Trypan Blue, were counted and incorporated into cell-density calculations.

3.5.1 CSE-stimulation of epithelial cell culture

CSE was prepared in accordance with procedures previously outlined (Grigg et al. 2012) and stored at -20°C as 100% stock solutions. CSE was diluted in culture media to a concentration of 1%, which has previously been optimized to facilitate a two-fold upregulation in PAFr expression (Grigg et al. 2012). BEAS-2B cells were exposed to 1% CSE solution for 4 hours, at 37°C, 5% CO₂.

3.5.2 Bacterial strains and growth conditions (for PAFr dependent adhesion assay)

Clinical isolates of *S. pneumoniae* (strain 132), and non-typeable *Haemophilus influenzae* (NTHi) (strain RHH3) were obtained from the respiratory tracts of patients at the Royal Hobart Hospital, Tasmania. NCTC-4560 (ATCC 19418), an NTHi reference strain, was also used in this study. All bacterial isolates were recovered from frozen stocks (-20°C) and cultured at 35°C, 5% CO₂ without shaking. *S. pneumoniae* was grown overnight in Brain

Heart Infusion broth (BHI, Oxoid, CM1135, USA) and diluted in fresh BHI to an optical density at 600 nm (OD_{600}) of 0.08. using a calibrated Eppendorf BioSpectrophotometer (Eppendorf, USA). Bacterial suspensions were incubated at 35°C, 5% CO₂ with measurements taken hourly until stationary phase was ascertained. The bacterial number was confirmed by plating serial dilutions of the starting inocula and performing colony-forming unit (CFU) counting after incubating for 24 hours at 35°C, 5% CO₂. NTHi isolates were grown on Chocolate Agar plates (Oxoid, CM0271, USA) and colonies were re-suspended in Phosphate Buffered Saline (PBS; Life Technologies, USA). Serial dilutions were plated onto Chocolate Agar for subsequent CFU determination. For each bacterial strain, a multiplicity of infection (MOI) in the range of 10-50 bacteria per BEAS-2B cell was used. We aimed to fully define the effect of CSE and PAFr antagonist on *S. pneumoniae* adhesion and PAFr in BEAS-2B cells, then investigate the other major pathogen, NTHi.

After overnight incubation, *S. pneumoniae*, and NTHi were individually suspended into PBS to achieve an OD_{600} nm of between 0.12 - 0.18 OD_{600} nm. The suspension was centrifuged at 6000 RPM for 5 minutes to create a cell pellet. The supernatant was then aspirated, with each bacterial pellet suspended in 2 mL of carbonate buffers containing Fluorescein isothiocyanate (FITC; 1 mg/mL, Sigma Aldrich, USA). For experiments with *S. pneumoniae*, FITC-carbonate buffers consisted of Sodium Carbonate (NaCO₂: 0.05 M) and Sodium Chloride (NaCl: 0.1 M) diluted in sterile deionised water (dH₂O). Following protocols outlined by Avadhanula et al., strains of NTHi were suspended in sterile dH₂O comprising Sodium Chloride (0.1 M), Sodium Carbonate (0.09 M) and Sodium Bicarbonate (Na₂CO₃: 0.015 M) (pH 9.2) (Avadhanula, Rodriguez, Ulett, et al. 2006). All bacteria were suspended in FITC buffer for 1 hour at 4 °C before being centrifuged at 6,000 RPM for 5 minutes and resuspended in PBS twice. This was done to ensure no free FITC was remaining in solution before the final bacterial pellet was added to BEGM lacking gentamicin, which was also chosen to ensure culture media did not affect bacterial viability. Thus, FITC-labelled bacteria in BEGM without gentamicin was inoculated into wells and incubated for 1 hour at 37 °C, 5% CO₂. To ensure FITC did not affect the viability of bacterial strains, serial

dilutions of the starting inocula were plated on Chocolate agar and retrospectively analysed in triplicate.

An infection assay was adapted from previous collaborations between the CRE Breathe Well Group and Grigg and colleagues at Barts and the London School of Medicine and Dentistry (United Kingdom) along with previous NTHi adhesion assays using the BEAS-2B cell line (Avadhanula, Rodriguez et al. 2006; Grigg et al. 2012; Mushtaq et al. 2011). Bacterial adhesion assays were performed on *S. pneumoniae* 132, and NTHi strains NCTC-4560 and RHH3 using sterile Millicell EZ-Slide 8-well glass plates (EMD Millipore, USA). Cell monolayers were exposed to several treatments, with comparisons made between untreated-BEAS-2B cells, CSE-treated cells, as well as untreated and CSE-treated cells inoculated with bacteria. The effect of adding PAFr antagonists prior to infection on bacterial adhesion to CSE-treated cells was also measured. All bacterial adhesion assays were run as three biological replicates.

3.5.3 Treatment with PAFr antagonists

WEB-2086 was dissolved in dimethyl sulfoxide (DMSO) and added to culture media at a 1:1000 dilution or less to ensure DMSO concentrations were less than 1%. Ten-fold serial dilutions of each compound were completed (10 μ M, 1 μ M, 100 nM, 10 nM) to determine the anti-infective properties of PAFr antagonist and stored as frozen stocks at -20°C before being warmed to 37°C prior to use. Cell monolayers were washed once with PBS for 5 minutes, prior to PAFr antagonist inoculation. Cells were then exposed to PAFr antagonist dilution and incubated for 1 hour at 37°C, 5% CO₂ before being carefully aspirated.

3.5.4 Fixation of cells and preparation of antibodies (anti-PAFr and anti-ICAM-1)

After exposure to bacteria, cells were washed in PBS and fixed with 4% paraformaldehyde (Sigma Aldrich, USA) for 20 minutes at room temperature

and rinsed in PBS for five minutes three times. Cells were treated with two five-minute washes of ice-cold acetone and aspirated. After blocking with 1% Bovine Serum Albumin (Oxoid, USA), 1% Triton-X-100 (Sigma Aldrich, USA) in PBS (PBST) for 30 minutes at room temperature, cells were incubated with a 1:100 dilution of anti-PAFr monoclonal antibody (Cayman Chemicals, USA) overnight at 4°C in the dark, or 1:100 conjugated anti-PAFr polyclonal antibody (Cayman, USA) for 90 minutes at room temperature in the dark. For ICAM-1 protein staining, fixed cells were incubated with an anti-ICAM-1 antibody (1/250).

After thorough rinsing with PBS, 1:500 dilution of AlexaFluor 594-conjugated Goat Anti-Mouse secondary antibody (Molecular Probes, USA) diluted in 1% BSA, PBST for one hour at room temperature was used on cells previously incubated with anti-PAFr monoclonal antibody (Cayman Chemicals, USA). Similarly, a 1/500 dilution of AlexaFluor 498-conjugated goat anti-mouse secondary antibody (Molecular Probes, USA) in blocking buffer for 1 hour at room temperature was used to illustrate anti-ICAM-1 monoclonal antibody.

After incubation, three five-minute PBS washes were completed before 4', 6-diamidino-2-phenylindole (DAPI; Life Technologies, USA) at a concentration of 1:5000 in PBS was incubated in the dark at room temperature for 15 minutes when using either anti-PAFr or anti-ICAM-1 antibody. The cells were washed three times with PBS before slides were mounted with Fluorescent-mounting Media (Dako, Denmark A/S).

3.5.5 Microscopy and image analyses

Micrographs were analysed using an Olympus BX50 Fluorescence Microscope (Olympus; Tokyo, Japan) with NIH elements microscopy software (Nikon; Tokyo, Japan) and CoolSnap Hq2 CCD camera (Photometrics, USA). Five images were taken per well using each immunofluorescence channel to take pictures of DAPI (eukaryotic cell nuclei; 30 ms exposure, 405 nm), PAFr (PAFr expression; 1-3 second exposure, 594 nm) and FITC (bacteria; 1-3 second exposure, 488 nm) signals using the 40× lens. NIH elements imaging

software (Nikon; Tokyo, Japan) was used to perform eukaryotic and bacterial cell counts, whilst image merging was completed using Adobe Photoshop C56 software (Adobe Systems, California, USA). Cell tracing was achieved using Image-Pro Plus 7.0 software (Media Cybernetics, Inc., USA).

3.5.6 BEAS-2B cell viability assay

Cells were passaged into sterile clear-flat 96-well plates (Sigma Aldrich, CLS3340, USA) with each PAFr antagonist diluted to concentrations used within bacterial adhesion assays and added as triplicates to incubate for 1 hour. Alamar Blue (Life Technologies, USA) was then added into each well at a 10% concentration before hourly absorbance readings (585 nm) until 24 hours using a Spectromax Spectrophotometer Microplate Reader (Molecular Devices, USA). A positive control of 100% reduced Alamar Blue via autoclaving was also used in experiments. Cell viability data is representative of three biological replicates and expressed as IC₅₀ values based upon calculations from manufacturer's guidelines (Life Technologies, USA).

3.5.7 In silico analyses of PAFr antagonist compound docking

I am grateful to Dr. David Gell (protein biochemist) and Mr. Rory L. Fairbairn (honors student) for performing these *in silico* analyses. To understand the binding of ligands to PAFr, a 3-dimensional molecular model of the PAFr protein was generated. The software package, MODELLER (Andej Sali, San Francisco, CA 94143, USA) was used to create a PAFr protein structure based upon the known X-ray crystal structures of related proteins (Caboni et al. 2014; Gazos-Lopes et al. 2014; Gui et al. 2007). The program takes as input: (1) an alignment of the PAFr amino acid sequence with sequence (or sequences) of G32 protein coupled receptor(s) of known structure; (2) the atomic coordinates of known structures in Protein Data Bank (pdb) format (Marti-Renom et al. 2000; Sali & Blundell 1993).

The software package, MODELLER (Andej Sali, San Francisco, CA 94143, USA) (Sali & Blundell 1993) was used to create a PAFr protein structure based upon the known X-ray crystal structures of other G-protein coupled

receptors (GPCRs), namely, neurotensin receptor type 1 (pdb 4buo, 2.75 Å resolution), bovine rhodopsin (pdb 1u19, 2.2 Å resolution), and an engineered nociception/orphanin FQ receptor (pdb 4ea3, 3.0 Å resolution). A second model was obtained using the I-TASSER web server and a database of structural models including all 1,026 GPCRs in the human genome (Zhang et al. 2015), which identified C-C chemokine receptor 5 (CCR5; pdb 4mbs, 2.7 Å resolution) as a template with significant similarity to PAFr. Ligand docking was performed using AUTODOCK VINA software (Molecular Graphics Laboratory, California, USA) (Trott & Olson 2010). Atomic coordinate files for WEB-2086 and PAF in Protein Data Bank (PDB) format were generated using OPEN BABEL (O'Boyle et al. 2011) and input files for docking were prepared using AUTODOCK TOOLS (Sanner 1999). Docking results were visualized and molecular graphics generated using PYMOL (Schrödinger, USA).

3.5.8 Quantitative real-time PCR (qPCR) for PAFr mRNA analysis

Cells were seeded into a sterile clear-flat bottom 24-well plate (Corning, USA). The unstimulated cells and cells exposed to CSE (1%, 4 hours). RNA was prepared using the Reliaprep™ Mini RNA cell Miniprep system (Promega, Australia) with total RNA quantified by Nanodrop 1000 (Thermo Scientific, USA). Complementary DNA (cDNA) was then collected via Promega cDNA synthesis kit (Promega, Australia). Transcript level of PAFr was assessed by a commercially available primer (Qiagen, Germany, Catalogue#PPH05820C), whilst ICAM-1 was assessed (H_ICAM1_1, Sigma Aldrich, USA) was determined by qPCR using the Corbett Rotor-Gene 6000 system (Qiagen, Germany). Reaction volumes in qPCR assay were 15 µL in 0.1 mL tubes containing 100 pg of template, 0.5 µL PAFr primer (10 mM, Qiagen, Germany), and 7.5 µL SensiFast SYBR (Bioline, BIO-98005, Australia). Thermocycling controls were run as previously described (Latham, Zhang & Tristram 2015). Briefly, this consisted of an initial melt at 95°C for 3 minutes before 40 cycles at 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 10 seconds. Data acquiring was completed at 72°C, with melt curve collection between 60°C and 95°C. The relative change of expression was normalised to all three-reference genes (18S, β-Actin, β2-Microglobulin) using

comparative analysis according to the manufacturer's guidelines (Qiagen, Germany). Data shown is representative of two separate experiments with two replicates.

3.6 Statistical analysis

For cross-sectional data, the distributions were generally skewed in an upward direction, so results are presented as medians and ranges; non-parametric analyses of variance were performed (Kruskal-Wallis Test comparing medians across all the groups of interest) and specific group differences were then explored as appropriate according to prior hypotheses using the Mann–Whitney U test. To be highly conservative, we have also applied a Bonferroni correction for multiple comparisons. We also performed regression analysis for PAFr expression against age, FEV₁ (%predicted), and smoking history in the normal smokers and COPD groups. Associations between variables were assessed using Spearman's rank test. Linear regression analyses for potential confounders were undertaken and differences between groups in gender balance, age, and atopy were found to be non-contributory. We also performed regression analysis for both PAFr and ICAM-1 expression against age, FEV₁, and smoking history in both the normal smoker controls and COPD patients and CAL groups separately. For in vitro studies, data are expressed as mean \pm standard error using Microsoft Excel Statistics package (Microsoft, United States of America). Comparisons between groups were completed using one-way analysis of variance (ANOVA), and unpaired two-tailed *t* tests using GraphPad Prism (GraphPad Software Inc., United States of America). In this work statistical analyses with $p < 0.05$ were considered significant.

Statistical analyses were performed using SPSS 21.0 for Mac, 2012 (IBM Corporation, Armonk, NY, USA) and GraphPad Prism 6.0 (2012) for Windows, (GraphPad Software Inc., La Jolla, CA, USA), with a two-tailed *p*-value ≤ 0.05 being considered statistically significant.

Chapter 4

Large airway epithelial platelet activating factor receptor (PAFr) expression is markedly upregulated in Chronic Obstructive Pulmonary Disease

4.1 Introduction

Emphasis has been laid on the importance of lower respiratory tract bacterial colonization in the pathogenesis of COPD, its subsequent long-term progression and acute exacerbations, and so infection is likely to be one of the important factors driving inflammatory processes in the airways (Cabello et al. 1997; Erkan et al. 2008; Patel et al. 2002). However, the mechanisms underlying the initial bacterial epithelial adherence and subsequent acute and chronic infections remain elusive. *Haemophilus influenzae* and *Streptococcus pneumoniae* are the most important pulmonary bacteria during both stable phase of disease and exacerbations of COPD (Banerjee, Khair & Honeybourne 2004; Murphy et al. 2004; Soler et al. 1999). Moreover, complex interactions between the host, microbes (both bacteria and viruses) and environmental pollution are associated with exacerbations that are marked by substantial increases in inflammatory markers in the airways (Hurst et al. 2006; Sapey & Stockley 2006).

Large intervention trials and follow-up pharmaco-epidemiology studies have recommended the use of inhaled corticosteroids (ICS) to reduce frequency of exacerbations and improve quality of life in more severe COPD patients, and it has become an established therapy (Calverley et al. 2007). Although ICS therapy undoubtedly has some positive effects, it is accompanied by an increase in the risk of lower respiratory tract bacterial infections in COPD patients, especially with *Streptococcus pneumoniae*, which increase in incidence over time (Cates 2013; Ernst et al. 2007; Singh, Amin & Loke 2009).

In an international collaborative study, our group recently demonstrated increased Platelet Activating Factor receptor (PAFr)-expression and PAFr-dependent *Streptococcus pneumoniae* adhesion to epithelial cells in culture exposed to cigarette smoke extract (CSE), and provided at least very preliminary evidence of an increase in airway epithelial expression of PAFr in normal smokers (Grigg et al. 2012).

PAFr is a G-protein-coupled epithelial cell membrane receptor that naturally binds the phosphorylcholine ligand on the eukaryotic pro-inflammatory chemokine PAF. Of all bacteria, *Streptococcus pneumoniae* (also *Haemophilus* and *Pseudomonas*) cell walls specifically express phosphorylcholine (ChoP), which out-competes PAF to bind to the PAFr. This initiates bacterial uptake by the host cell in a β -arrestin-dependent pathway (Cundell et al. 1995; Radin et al. 2005; Swords et al. 2000).

In addition, PAFr-deficient mice are less likely to develop invasive pneumococcal infections as compared to their wild-type counterparts (Rijneveld et al. 2004). PAFr-dependent pneumococcal adhesion is also upregulated by several other factors in addition to cigarette smoke, including acid, respiratory viral infection, interleukin (IL)-1 α and exposure to particulate matter (Grigg 2012). This all fits with the pattern of infection and deterioration seen in COPD.

In this chapter, I have extended our previous cross-sectional observations, to analyse the expression of airway epithelial PAFr in another batch of normal smoker tissue, but also in groups of COPD subjects (smokers and ex-smokers) not on ICS. I also took the opportunity to assess PAFr expression in a group of similar COPD subjects who had been involved in a trial of ICS therapy for 6 months (see page 74).

4.2 Overview of materials and methods

The subjects involved are detailed in pages 74-75. We employed classical immunohistochemical methods to evaluate PAFr expression in the large

airway epithelium in bronchial biopsies collected from well phenotyped participants (detailed in chapter 3, page 74-82).

CONSORT 2010 flow diagram

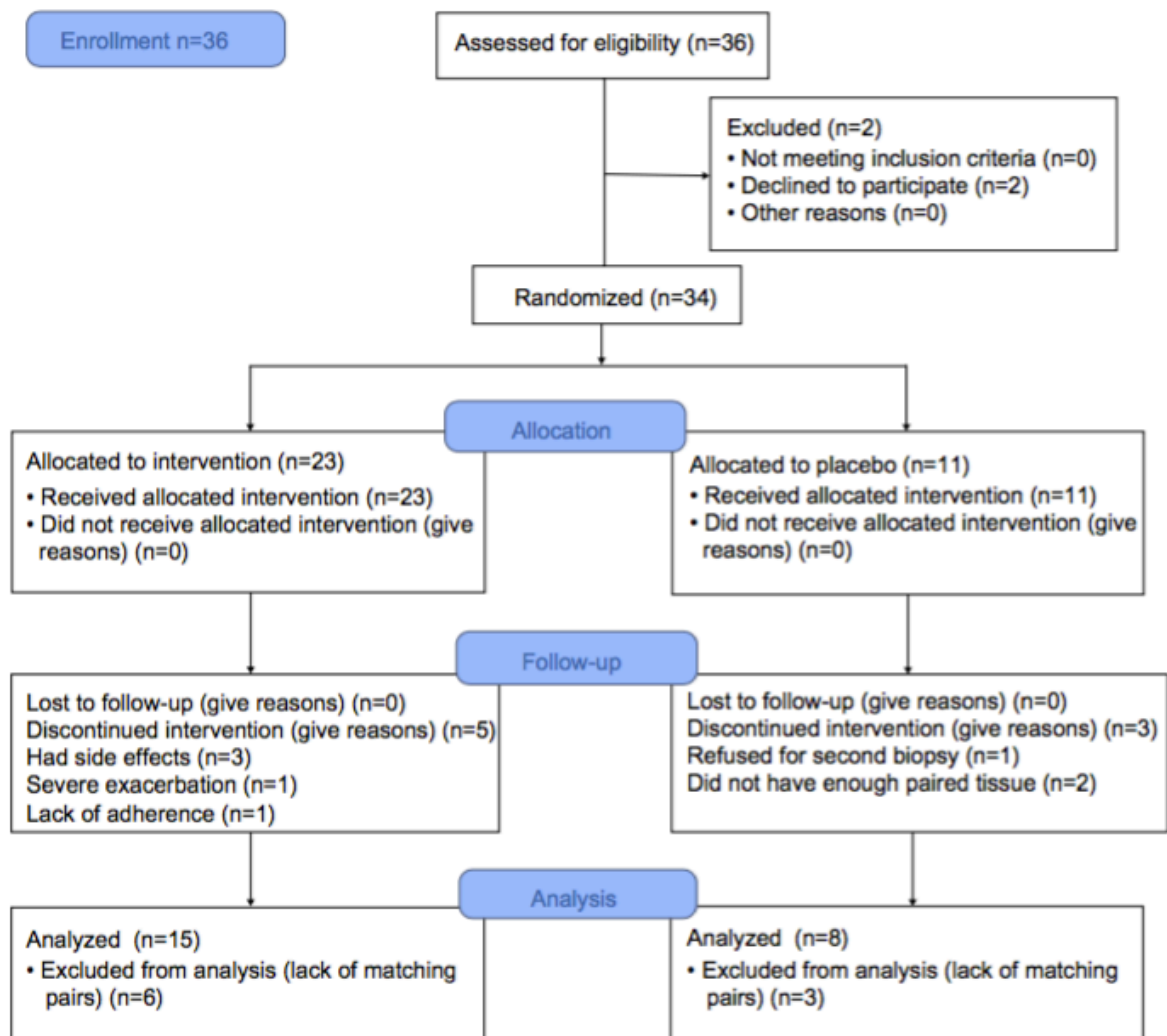


Figure 4.1. Study design for ICS intervention study.

Thirty four COPD patients; two week run-in period; then bronchoscopy and airway biopsy; then patients randomized 2:1 into receiving fluticasone propionate or placebo for 6 months by using a computer generated random-numbers table; finally, bronchoscopy and airway biopsy repeated. Tissue analysis done blinded to subject status (n=6 in the intervention arm and n=3 in placebo arm were excluded from analysis due to lack of tissue at second time-point).

4.3 Results

4.3.1 PAFr expression in cross-sectional study

Immunostaining was prominent only in the apical part of the bronchial epithelium of NLFS, but staining was darker and more generalized, apparently around the perimeter of cells, in the COPD groups. There was negligible PAFr staining in normal controls. These staining patterns are illustrated in Figure 4.2.

Image analysis confirmed that there was no difference in PAFr expression between COPD-smokers and COPD-ex smokers study groups ($p=NS$), but the difference between both COPD groups, and NLFS (Median 0.0%, Range 0-4.9%) was significant, i.e., COPD-smokers ($p<0.005$; Median 1.03%, Range 0-21.8%), COPD-ex smokers ($p<0.002$; Median 3.6%, Range 0-15.5%). NLFS also showed significantly higher PAFr when compared to NC (Median 0.0%, range 0-0%). PAFr expression in the ICS treated group was very similar to the other COPD groups, so no formal statistical testing was done (Figure 4.3). In the NLFS group, only 7 of the 16 epithelia stained detectably, while in the two COPD groups combined, 24 had detectable staining out of 27 (X^2 test; $p<0.001$), as five samples from COPD groups (2/17 COPD-smokers and 3/15 COPD-ex smokers) did not have enough tissue for analysis.

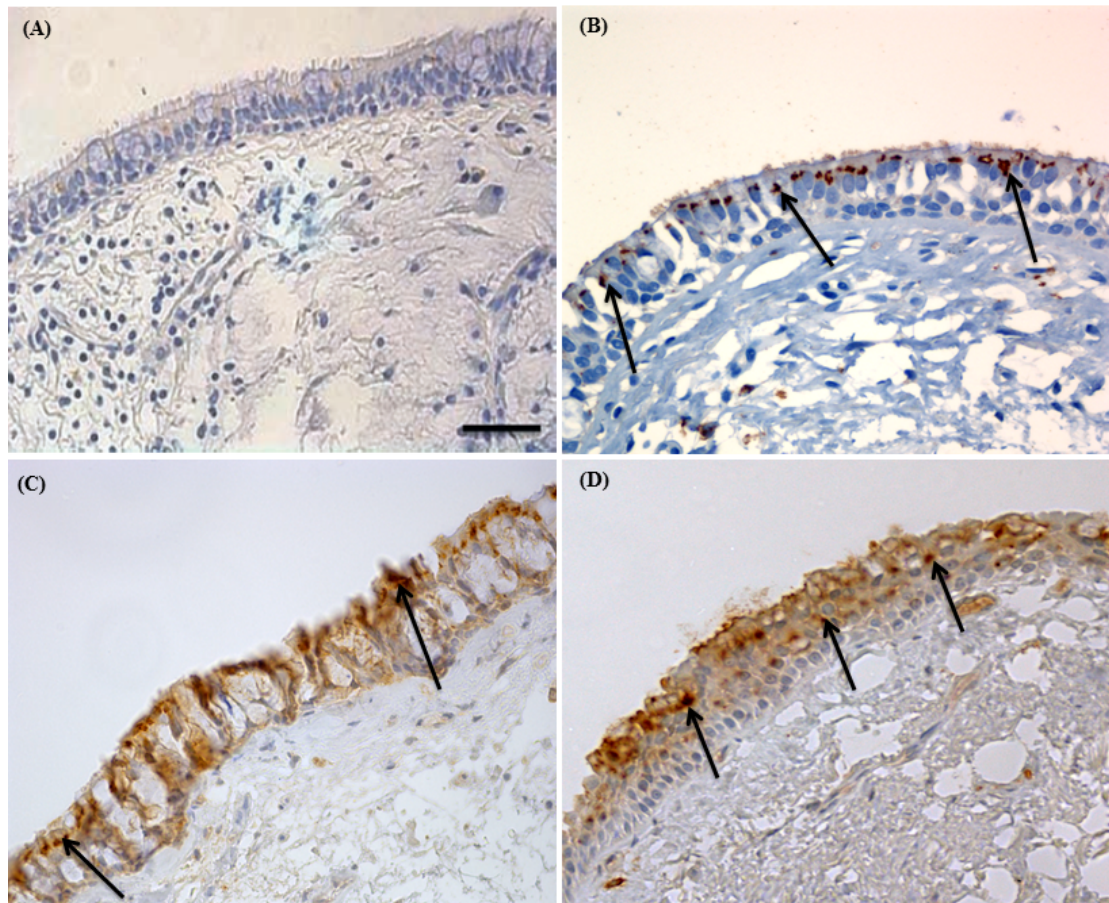


Figure 4. 2. Platelet activating factor receptor (PAFr) expression in epithelium of large airways.

(A) A representative bronchial biopsy from a never smoker showing low PAFr staining that is below the cutoff threshold for positivity (Republished with permission from BMJ Publishing Group Ltd [18]). (B) Typical lung function smoker, showing positive staining (arrowed). (C) Typical COPD- smoker showing extensive PAFr staining. (D) Typical COPD- ex smoker showing brown staining (arrowed). Original magnification x400. Scale bar = 50 μ m.

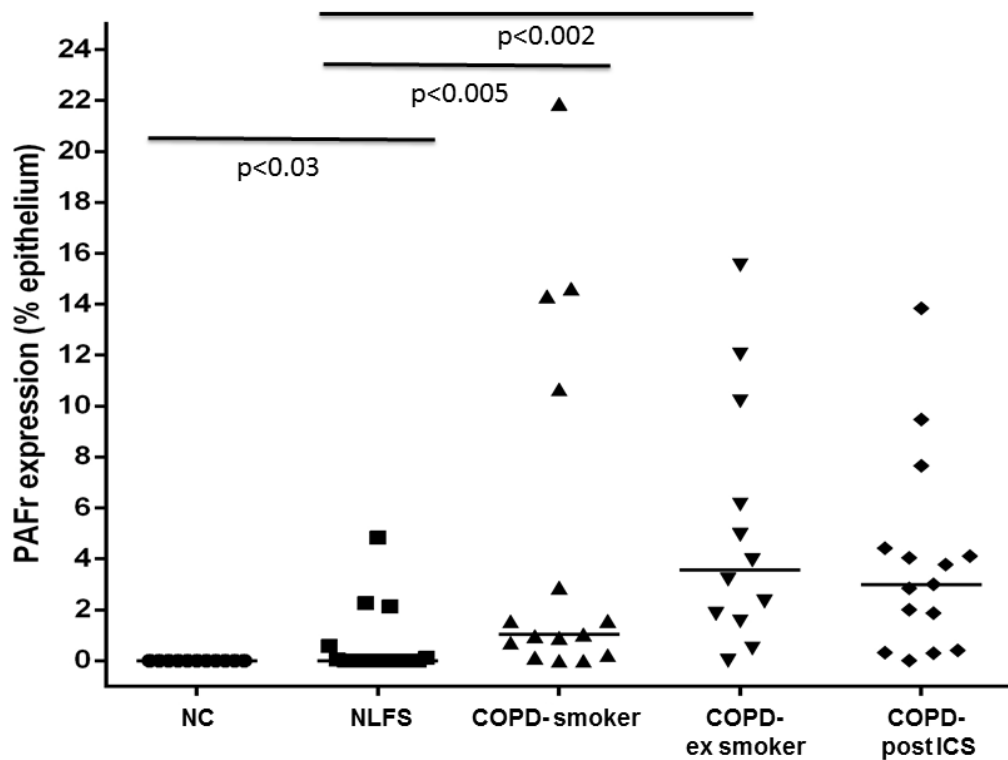


Figure 4. 3. Percentage of epithelium showing PAFr expression in cross-sectional study.

The COPD group after ICS is showed for completeness and is obviously quite similar to the COPD groups. The p values include a Bonferroni correction ($p < 0.001$). Horizontal Bars represent median.

The regression analyses showed that age and FEV₁ (% predicted) had no significant effects on PAFr-expression ($r=0.23$ and $r=0.11$ respectively). However, for pack-years of smoking in the ex-smoking COPD group, there was quite a strong relationship ($r=0.56$; $p<0.05$), but this was not seen in the currently smoking COPD subjects ($r=0.09$; $p<0.81$) (figure 4.4).

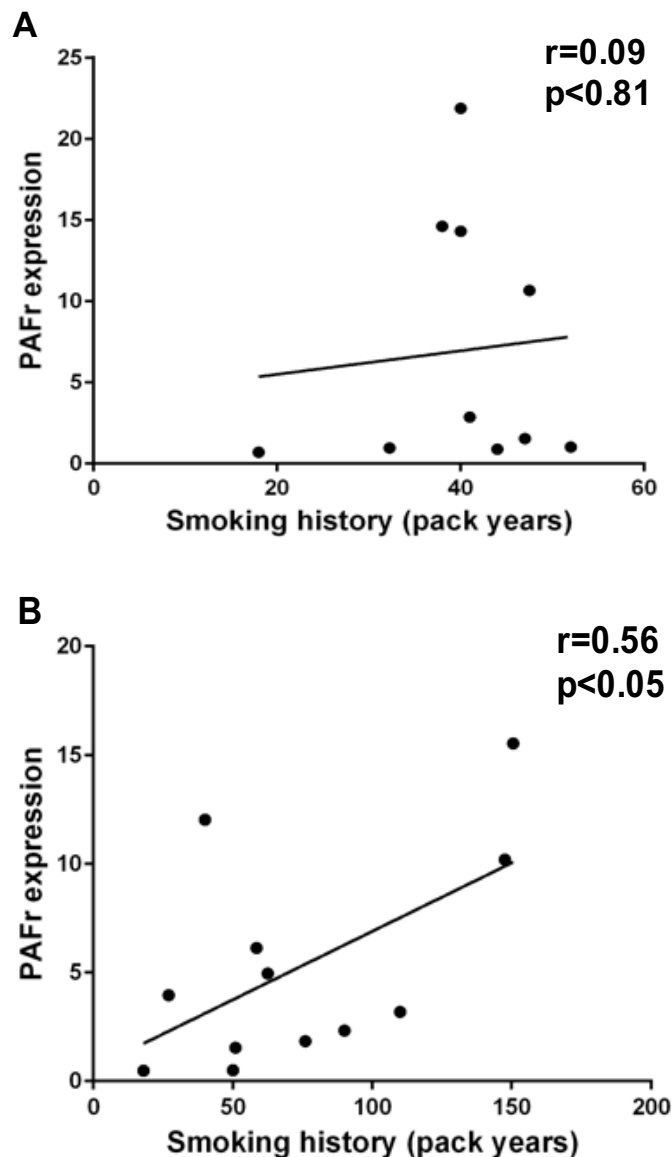


Figure 4.4. Correlations of epithelial PAFr expression in COPD groups and smoking history in pack-years.

(A) COPD-smoker. (B) COPD-ex smoker.

(pack years = Number of pack of 20 cigarettes each smoked on average per day x number of years of smoking).

4.3.2 PAFr expression in central airways obtained in a placebo-controlled ICS intervention study

There was considerable variation in PAFr expression within the COPD groups both before and after the treatment phase. The median percent epithelial expression rose in both the groups, by approx. 3-fold in the ICS limb ($p<0.15$) but also approx. two fold in the placebo group over time (figure 4.5). Although the final median level in the ICS groups was greater than in the placebo group, this was neither significant nor were the changes in expression over this time between the groups ($p<0.4$). It is possible, because of the small number of paired tissues that were available to us, that we are seeing a Type-2 statistical error and so a power calculation using non-transformed data was undertaken for a potential real increase in epithelial PAFr expression with ICS, which indicated that for these estimates of effect size, a bronchoscopic study of over 200 subjects per group would be needed to show a significant positive effect of ICS on PAFr expression.

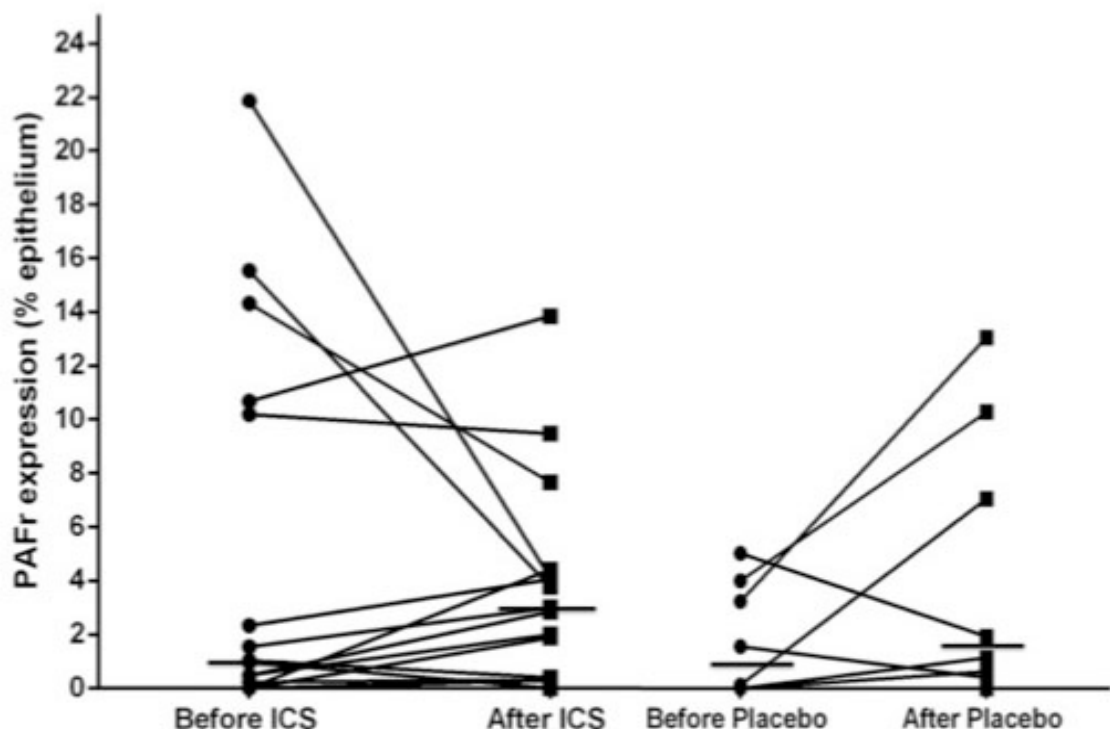


Figure 4.5. Relative PAFr expression in the ICS intervention study.

Horizontal Bars represent median.

4.4 Discussion

In the present study, I found that in vivo expression of PAFr is significantly upregulated in the central airways in COPD patients as compared to smokers with normal lung function and even more when compared to normal individuals. Expression in COPD current and ex-smoker was similar, but quite variable in both groups. This might suggest that smoking cessation may be ineffective in down-regulation of PAFr once COPD has developed. Alternatively, it is possible that especially high expression of PAFr in some smokers is both a risk factor for COPD development in the first place and its progression even after smoking has ceased.

Overall, my data on the effects of ICS were negative, with at the very most only modest evidence that ICS up-regulates PAFr expression, though it certainly does not decrease it over 6 months, as was suggested in earlier in vitro and animal models (Barbier, Agusti & Alberti 2008). This study may have been relatively short to demonstrate relevant effect of ICS on PAFr expression, as a 2-year timescale has usually been taken in monitoring Pneumococcal disease in COPD patients treated with ICS. Thus, incidence of pneumonia in patients on ICS escalates over years rather than weeks or months (Cates 2013). It is also possible that the effects of ICS could be more marked in peripheral airways or lung parenchymal epithelium where the clinical problem of pneumonia occurs while our biopsies were taken only from relatively central airways. Pneumonia occurs at a rate of approximately 1% per year in severe COPD patients on ICS (Kew & Seniukovich 2014), and it is not known whether there is a specific aberration only in this small group of individuals affected, or whether some generic change occurs in all COPD subjects given ICS but which manifests clinically only in a small percentage. This issue needs further work.

The relationship between cigarette smoking and infection/colonization with *Streptococcus pneumoniae* and NTHi has been well documented and exposure to tobacco smoke is considered as a major risk factor for invasive pneumococcal disease (Arcavi & Benowitz 2004; Nuorti et al. 2000).

Persistence of chronic inflammation and continuous deterioration of lung function following smoking cessation may be attributed to chronic colonization and invasion of lung tissue by respiratory bacterial pathogens, again most commonly *S. pneumoniae* and *H. influenzae* (Arcavi & Benowitz 2004). Bacterial adherence to lung epithelium and subsequent tissue invasion is thought to be a crucial step in the pathogenesis of respiratory infections. The major respiratory bacterial pathogens are equipped with the surface ligand, a mimic of platelet activating factor (PAF), known as phosphorylcholine (ChoP), which is capable of binding to epithelial PAFr. Especially virulent strains of *S. pneumoniae* have been shown to adhere its bacterial cell surface ChoP especially firmly with epithelial surface PAFr (Cundell et al. 1995; Grigg 2012; Swords et al. 2000). I suggest that my data on PAFr expression in the airway epithelium in COPD may be relevant to this, as out of an estimated 10^9 different bacterial species (Curtis, Sloan & Scannell 2002), it is only these two species particularly (and also *Pseudomonas aeruginosa*, which is more relevant in cystic fibrosis) that express the ChoP ligand.

In a collaborative study with Grigg et al. (Grigg et al. 2012), our group has described significant increased in PAFr-dependent pneumococcal adhesion, PAFr transcript level and PAFr protein expression in epithelial cell cultures exposed to cigarette smoke extract (CSE). Moreover, a specific PAFr antagonist attenuated pneumococcal adhesion. In the same study we showed enhanced airway epithelial PAFr expression in smokers airways for the first time. My study takes this observation further and confirmed much greater up-regulation of PAFr expression in the COPD groups than in normal smokers. In contrast, Suri et al. reported no difference in PAFr mRNA transcript levels between normal smokers and smokers with COPD. However, they did not quantify the PAFr immunoreactivity on bronchial biopsies, and ultimately it is protein expression on the epithelial surface that will matter functionally (Suri et al. 2014), which might reflect post-transcriptional modification changes rather than transcription control. Moreover, the micrograph provided by Suri et al. seemed to have more PAFr expression in their normal controls than we have found, but the most staining did actually appear to be in COPD, consistent with our finding. These data raise the need for ex-vivo epithelial cultures from

such patients to see if functionally there is greater bacterial adhesion in COPD airways in the group with greatest PAFr expression.

The regression analysis in the two cross-sectional COPD groups produced interesting outcomes. There was no relationship between either age or severity of airflow obstruction and PAFr-expression, but pack-years of smoking was strongly related, at least in COPD patients who no longer smoked. The lack of this relationship in current smokers suggests that this aggregate long-term effect was confounded by an additional effect of current smoking. Thus, both current smoking and history of smoking seem important, but since only a minority of smokers develop COPD, there must also be in addition an individual susceptibility, presumably at a genetic or epigenetic level.

The role that inhaled corticosteroids (ICS) can play in COPD has been extensively studied in recent years and ICS have been shown to be especially effective at reducing exacerbation rates (Calverley et al. 2007). Other described benefits are more tentative, such as reduction in rate of decline in lung function, decreased cancer rates (Celli et al. 2008; Parimon et al. 2007) and decreased mortality (Calverley et al. 2007). However, a paradoxical effect is that ICS therapy also increases the risk of lower respiratory bacterial infections, especially with *S. pneumonia* (Calverley et al. 2007; Singh, Amin & Loke 2009), an effect that worsens over time (Cates 2013). An association between ICS and increased risk of hospitalizations due to pneumonia in elderly COPD patients with a high mortality rate has also been reported (Ernst et al. 2007). Animal models also demonstrate a similar effect of elevated risk of pneumonia with administration of ICS (Patterson et al. 2012).

On the basis of my current data, I cannot say that this increased infective risk of ICS is related to enhanced expression of respiratory epithelial PAFr, but it remains a possibility. A much larger clinical study would be needed to determine this, but such a large bronchoscopic investigation is rather unlikely to occur. A better approach may be to see if corticosteroids have any effect on human primary epithelial cell cultures in this regard. It is notable that PAFr

expression seemed to increase in both ICS and placebo treated COPD subjects over 6 months. Patients are recruited into such studies when especially well and free from infection, and this apparently spontaneous change may be a regression back to their more usual status of increased vulnerability.

There were several limiting issues in this study, especially the small numbers of samples available in the ICS intervention study. Secondly, only large airways were studied, but the next chapter extends my analysis to small airway samples and alveolar epithelium obtained at lung resection. Strengths of the present investigation include fairly robust numbers in the main cross-sectional study that followed on from the rather small smoking effect published previously using this same tissue bank (Grigg et al. 2012). The data for the normal lung function smokers analysed here were obtained de-novo and by me, as a completely separate operator, but are very similar to that published earlier

4.5 Conclusion

In summary, airway epithelial PAFr expression is significantly up-regulated in smokers but is especially marked in COPD subjects, irrespective of whether they are current smokers or not. This opens up a very significant potential to explain the pathogenesis and evolution of COPD, but it now needs further study, especially clinical follow-up. However, PAFr expression could be very important in the etiology of chronic and invasive infection with *Streptococcus pneumoniae* and *Haemophilus influenzae*. Whether the increased expression of PAFr is a contributing cause of COPD or a result of it especially needs further investigation.

Chapter 5

Platelet-activating factor receptor (PAFr) is upregulated in small airways and alveoli of smokers and COPD patients

5.1 Introduction

As discussed earlier, the most common “colonizers” in the stable state of COPD are *S. pneumoniae* and NTHi (Chapter 2). The risk of mortality and morbidity escalates when patients encounter frequent AECOPD, which are mostly of microbial origin.

In the previous chapter, I showed significantly increased expression of PAFr in the epithelium of central airways of both COPD-smokers and COPD-ex-smokers compared to smokers with normal lung function, which was found to be independent of age or FEV₁. I did find a strong relationship between PAFr-expression and pack-years of smoking in the COPD ex-smokers suggesting that smoking cessation is ineffective in affecting this chronic phase of PAFr upregulation once COPD has developed. Alternatively, it is possible that high expression of PAFr in some smokers may be a risk factor for development of COPD in the first place (chapter 4).

PAFr-protein expression in small airways has not previously been explored. Published evidence has established that the small conducting airways (<2mm in diameter) are the primary site of the majority of smoking-related airways obstruction in COPD (Burgel 2011; Cosio, Saetta & Agusti 2009). However, the extent and heterogeneity of pathological processes in small airways are yet to be fully understood, including the effects of smoking on the regulation of microbial adhesion sites, including PAFr. Since both *S. pneumoniae* and NTHi utilize PAFr for host epithelial adhesion, its upregulation in small airways could be especially important for inducing chronic infection and secondary inflammation and remodeling at this site.

In the present study, I have investigated whether PAFr expression is upregulated in epithelium of small airways and alveoli in smokers and patients with COPD, as well as a group with only small airway obstruction.

5.2 Overview of materials and methods

I employed classical immunohistochemical methods to evaluate PAFr expression in the large airway epithelium in bronchial biopsies collected from well phenotyped participants (as detailed in methodology chapter 3, page 77-83).

5.3 Results

5.3.1 Small airway epithelial PAFr expression

Epithelial expression was increased in NLFS ($p < 0.01$) compared to normal, but most markedly increased in both COPD-smokers and COPD-ex smokers (figure 5.1 A, B, C & figure 5.2A). Moreover, there was significant upregulation in PAFr expression in COPD smokers ($p < 0.05$) when compared to NLFS. In NLFS, epithelial staining was mainly apical, but in the other groups, heavy PAFr expression was observed throughout the airway epithelium. For all current smoker groups, together or separately, there were positive relationships between pack-year smoking history and PAFr expression ($r \geq 0.5$), strongest in the normal lung function smokers ($r = 0.9$; $p < 0.002$) (figure 5.3).

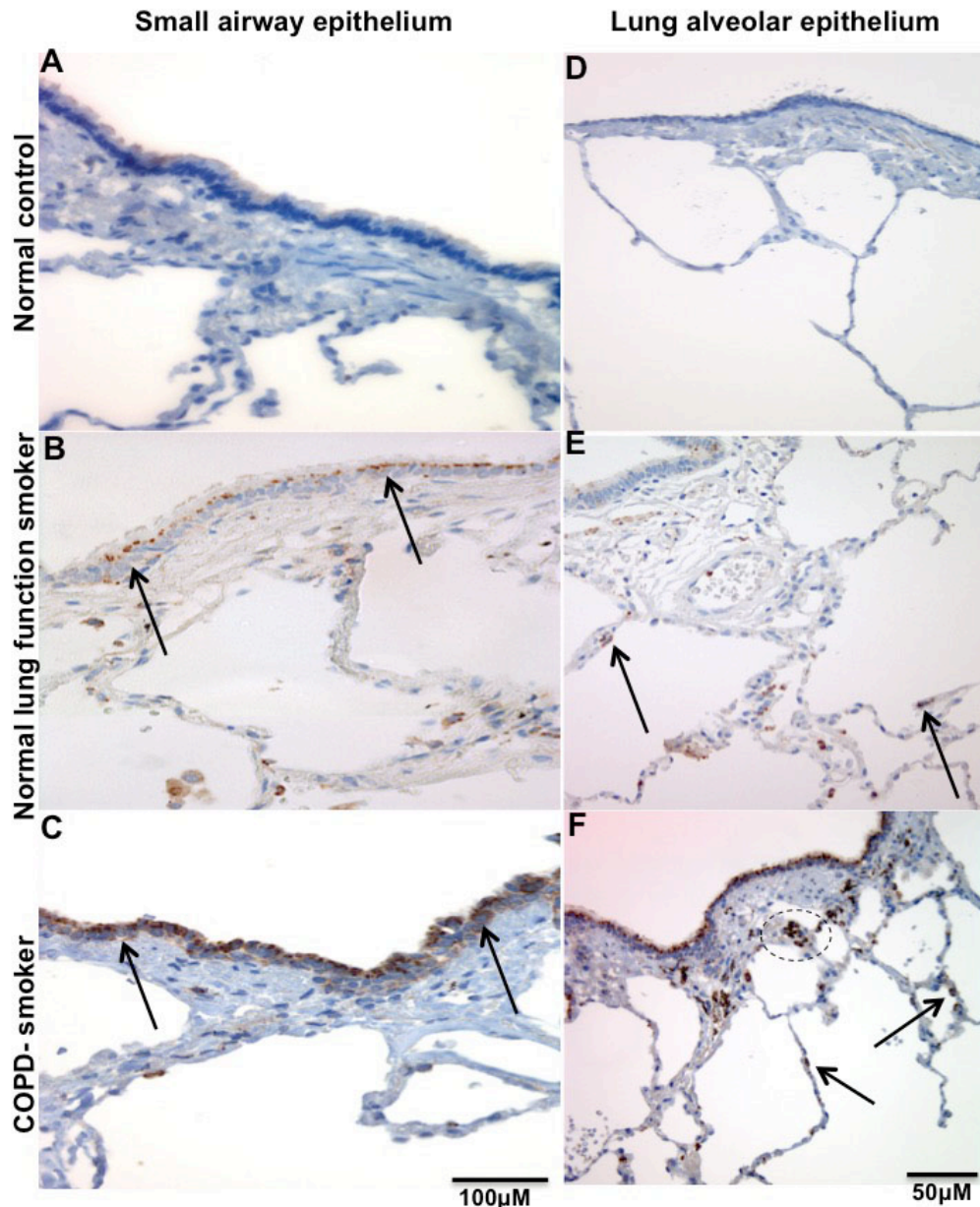


Figure 5.1 Platelet activating factor receptor (PAFr) expression in epithelium of small airways and lung parenchyma.

(A-C) and lung alveoli (D-F). (A,D) Representative section of small airway from a never smoker showing negligible PAFr staining that is below the cut off threshold for positivity. (B,E) Typical normal lung function smoker showing positive staining (showed by black arrow). (C,F) Typical COPD-smoker showing extensive PAFr staining (arrowed; black circle in F shows carbon deposition). A-C: Magnification x400; D-F: Magnification 200x. The small airway disease group stained similarly to COPD.

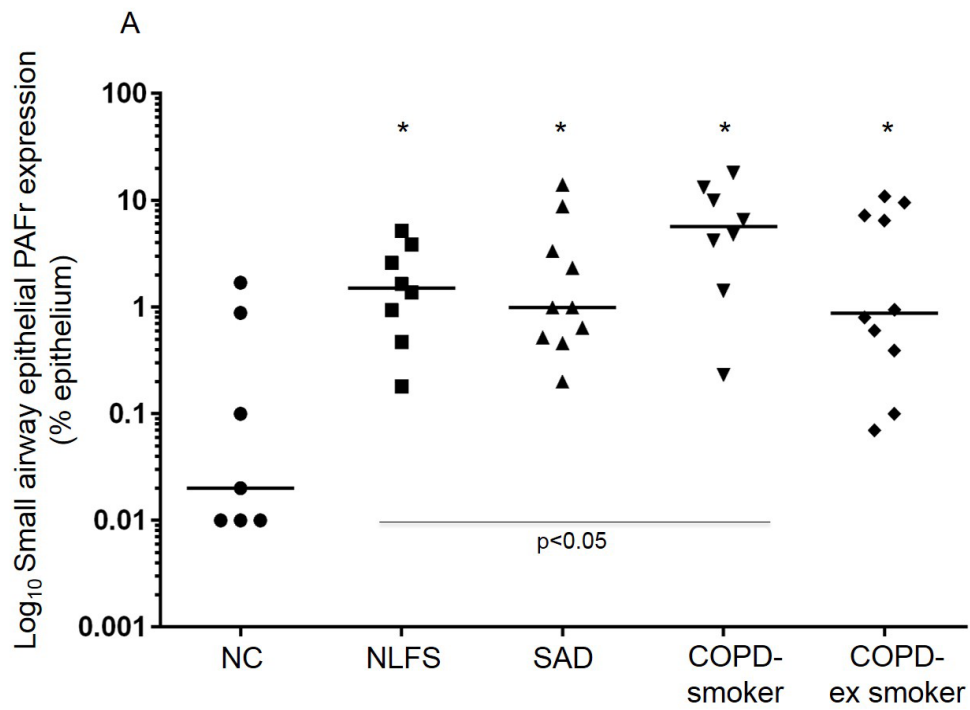


Figure 5.2. Percentage of small airways epithelium showing PAFr expression in cross-sectional study.

Data were log-transformed so the differences between groups are potentially large.

*p<0.01 compared to NC.

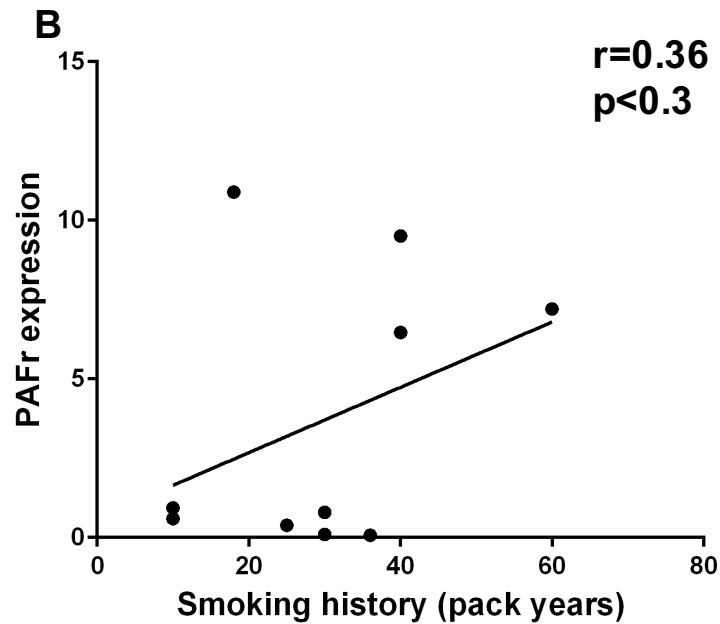
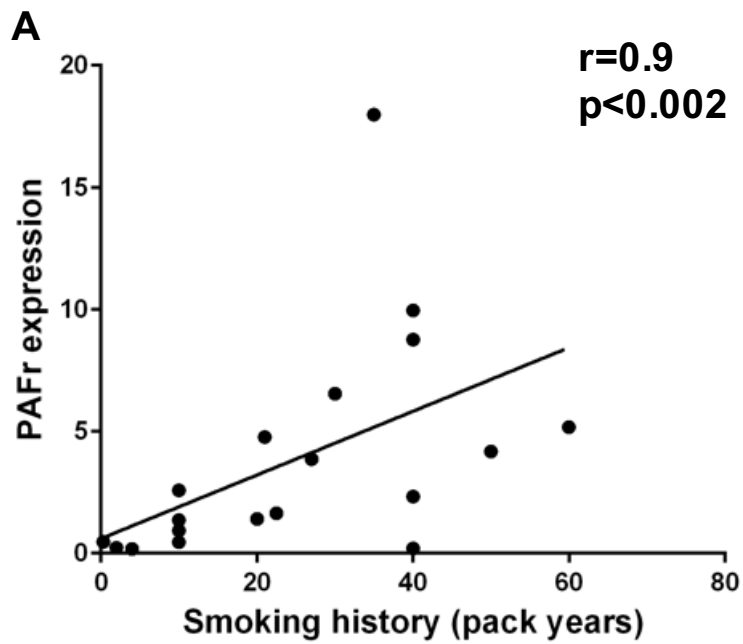


Figure 5.3. Correlations between epithelial PAFr-expression in small airways and smoking history in pack-years.

(A) for all currently smoking individuals. (B) COPD-ex smokers.

5.3.2 PAFr positive cells in the airway Rbm

The Rbm in both COPD groups was relatively hyper-cellular compared to normal, as previously observed (Sohal et al. 2010). The only significant increase in PAFr stained cells was observed in COPD-smokers ($p < 0.007$; median: 1.0 PAFr positive cells/mm of Rbm; range: 0.7-10.3), compared to normal tissue (figure 5.4).

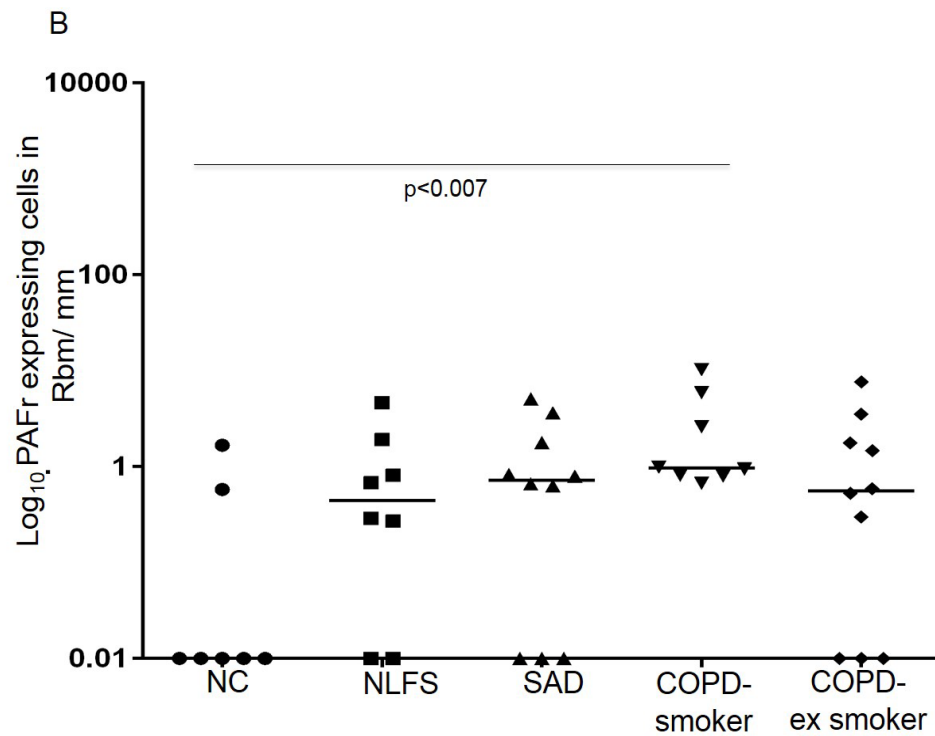


Figure 5.4. PAFr-expressing cells in Rbm of small airways in cross-sectional study.

5.3.3 PAFr positive cells in the alveolar epithelium

PAFr positive cells were significantly increased ($p < 0.01$) in all the clinical groups compared to normal tissue. Overall, approximately 15-20% of total epithelial cells (both type I and type II pneumocytes) were found to be positive across all the disease groups (figure 5.1 D, E, F & figure 5.5). The percentage of PAFr expressing cells in alveoli of normals was negligible.

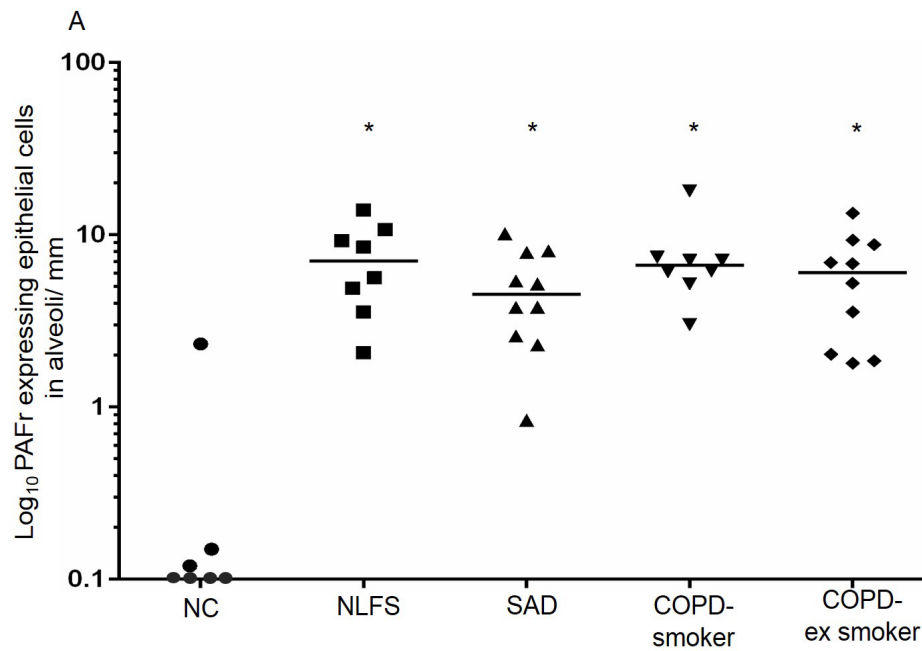


Figure 5.5. Quantification of PAFr-expressing cells in lung alveolar epithelial cells. All comparisons verses NC.

5.3.4 PAFr positive inflammatory cells in the lung alveolar interstitium

PAFr expression was present on inflammatory cells, even in the normal controls, mainly on macrophages, but these were always lightly stained. There was no statistically significant increase in either COPD group compared to normal. However, the number of PAFr-expressing macrophages was significantly increased in the interstitium in normal lung function smokers compared to normal tissue ($p < 0.008$) (figure 5.6).

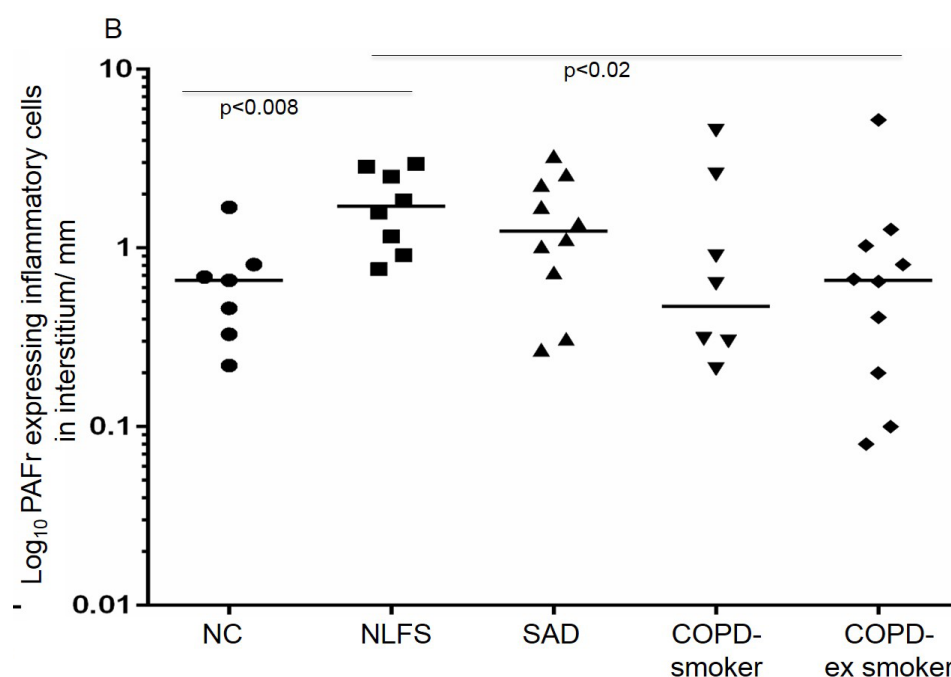


Figure 5.6. PAFr-expressing inflammatory cells in interstitium.

Data was log-transformed so the differences between groups are potentially large.

5.4 Discussion

This is the first comprehensive report of PAFr-protein expression in the epithelium of the small airways and lung parenchyma in smokers and patients with COPD. I found increased PAFr expression in the epithelium of both small airways and alveoli in smokers, but especially in the small airways in COPD

patients. I also found that the number of PAFr positive cells increased in the reticular basement membrane in COPD-smokers; most of these cells had fibroblast-like phenotype, as reported previously by our group in investigation of EMT in smoker airways (Sohal, S. S., Mahmood & Walters 2014; Sohal, S. S. et al. 2014). These findings could be crucial for understanding the vulnerability of smokers to airway infections specifically with NTHi and *S. pneumoniae*.

Increased expression of PAFr in smokers' large airway epithelium has been previously reported by Grigg et al. (Grigg et al. 2012). Previous evidence has indicated that environmental pollution (Mushtaq et al. 2011), acid exposure (Ishizuka et al. 2001), infections (both viral and bacterial) (Ishizuka et al. 2003), cytokines (IL-1 α and TNF- α) (Cundell et al. 1995) could all elevate epithelial PAFr expression, which is also associated with increased pneumococcal invasion in both cultures and animal models (Iovino et al. 2013; Sohal et al. 2014). Epithelial expression of PAFr in the small airways of smokers and COPD smokers is comparable to my previous findings in large airways (chapter 4; page 95-98), suggesting a smoking effect on upregulation of PAFr throughout the airway tree, but intensified in smoking-related obstructive disease.

The regression analysis in the smokers and COPD groups produced interesting outcomes. I did not find any relationship between either age or severity of airflow obstruction in COPD groups and small airway epithelial PAFr-expression. However, pack-years of smoking were strongly related to PAFr protein levels in all current smokers. These observations in small airways are quite different from those in the large airways (presented in chapter 4). The study populations were different between the two studies, which could account for these variations but since the inclusion criteria were similar this is unlikely. Thus, both current smoking and a history of smoking seem important for epithelial PAFr expression.

The importance of PAFr in chronic epithelial infection and invasion is documented in detail in other models, which is extensively discussed in

chapter 2 (pages 44-55). Also, bacterial ChoP interaction with host epithelial PAFr is likely to be important for both bacterial adherence and then invasion of host cells and tissues (Weiser, Shchepetov & Chong 1997). The interaction between this bacterial wall ligand (ChoP) and epithelial surface PAFr seems to be quite specific as only pathogens expressing ChoP are able to 'anchor' to PAFr. In addition to the role of PAFr in increasing the risk of infection, host PAFr and its activation has been shown to potentially enhance lung cancer progression and so may be implicated in some major features of COPD, i.e. airway infection and malignancy (Hackler et al. 2014)

I also observed PAFr expression on the surface of macrophages, and whether these receptors may possibly provide shelter to pathogens against inflammatory attack needs to be investigated. I did not find significant increase in PAFr positive inflammatory cells (phenotypically macrophages) in the alveoli of COPD groups compared to normal, as reported by others (Barnes 2008). However, there was an increase in physiologically normal smokers, consistent with recent reports in large airways (Sohal, S.S. et al. 2015), potentially due to an activated innate response to pathogenic bacteria.

Previous animal studies suggest PAFr to be a putative target for non-antibiotic therapies for treating COPD exacerbations by preventing specific binding of *S. pneumoniae* and *H. influenzae* to the respiratory epithelial surface (Cundell et al. 1995; Grigg et al. 2012; Swords et al. 2000). Existing PAFr antagonists were developed as potential anti-inflammatory agents in treating asthma but were not found to be effective. The potential role of PAFr-antagonist in reducing bacterial adhesion to respiratory cells in a PAFr-dependent manner will be discussed in the next chapter.

The strengths of the present study include the use of relevant human tissue, with fairly robust numbers in our cross-sectional study, following up from my previous observation (chapter 4) that assessed PAFr expression in large airways only. I have only analyzed mild to moderate COPD patients, as I wanted to look at pathogenic mechanisms in early disease. I assessed epithelium of both small airways and alveoli to get a comprehensive overview

of the respiratory tract. There are also a few limitations to this study. Firstly, it is cross-sectional at a single time point and longitudinal studies are required to see how variable the PAFr expression is even within individuals and if it relates to the natural history of smokers' airway disease and likelihood of AECOPD. I have analyzed PAFr-expression only at the final protein level so far, though ultimately that is what counts. Secondly, the control subjects were relatively younger, but their ages overlapped with our clinical samples. There was no relationship between PAFr expression and age in any group.

In conclusion, epithelial PAFr expression is upregulated in both small airways and alveoli in smokers, but is especially increased in small airway epithelium in COPD-smokers. There is a uniform increase in PAFr expression in alveolar epithelium in smokers/COPD but not in lung interstitial inflammatory cells. Increased expression of PAFr in the respiratory tract could be a crucial risk factor for acute and chronic infections with specific respiratory bacterial pathogens in smokers and especially in COPD. Translational research in this area is still in its infancy but has huge potential to modify clinical management of COPD, and indeed open up a new anti-PAFr therapeutic avenue.

Chapter 6

An antagonist of the Platelet-Activating Factor receptor inhibits adherence of both non- typeable *Haemophilus influenzae* and *Streptococcus pneumoniae* to cultured human bronchial epithelial cells exposed to cigarette smoke

6.1 Introduction

As mentioned in chapter 1, tobacco smoking is the most important causative factor for COPD (Fletcher and Peto 1977; Lokke et al. 2006; Lundback et al. 2003; Taylor 2010). Exposure to cigarette smoke has been shown to significantly increase invasive respiratory pneumococcal infections (Nuorti et al. 2000). Moreover, chronic exposure of mice to cigarette smoke resulted in significantly higher loads of NTHi and *S. pneumoniae* in the lungs (Voss et al. 2015).

In chapters 4 and 5, I have shown that PAFr expression is significantly upregulated in the airway epithelium of both the large and small airways in smokers and COPD patients. NTHi and *S. pneumoniae* may adhere to PAFr expressed on the luminal surface of human respiratory tract epithelial cells through physico-chemical interactions with phosphorylcholine (a molecular mimic of platelet-activating factor) on the cell wall surface of these particular bacteria.

As discussed in chapter 2 (page 44-55), Cigarette smoke has been shown to induce increased expression of PAFr in lower airway epithelial cells, which correlates with enhanced adhesion of *S. pneumoniae* (Grigg et al. 2012). In this work, I further investigated PAFr as an anti-infective therapeutic target and tested the effect of a well-characterized PAFr antagonist, WEB-2086 (Cellai et al. 2006; Cellai et al. 2002; Kato, M et al. 2004), for its potential to inhibit bacterial adhesion to respiratory epithelial cells by both NTHi and *S. pneumoniae*. This may provide a novel approach for the development of specific inhibitors of PAFr-bacterial interactions. This area needs urgent

attention as it could transform the therapeutics of COPD-related airway infection as a driver of disease progression.

6.2 Overview of materials and methods

I employed *in vitro* epithelial cell culture, quantitative real-time PCR (qPCR), immunofluorescence microscopy to evaluate PAFr expression and bacterial adhesion to both CSE treated BEAS-2B cells and controls. I am grateful to Mr. Rory L. Fairbairn, who assisted me carrying out this work. BEAS-2B bronchial epithelial cells were selected for use in this work based on their previous application in the study of PAFr expression in response to cigarette smoke exposure (Grigg et al. 2012). I assessed the effect of a specific, commercially available PAFr-antagonist on the adhesion pattern of bacterial strains to both CSE-treated BEAS-2B cells and control cells. Moreover, I worked with Dr. David Gell to carry out *in-silico* analyses to investigate the specific binding site of PAF (natural ligand of PAFr) and PAFr, and also the specific PAFr-antagonist (WEB-2086) to assess if the compound could be enhanced for better binding and increased inhibition of ChoP expressing bacterial strains. The specific methods are detailed in methodology section (chapter 3, page 83-91).

6.3 Results

6.3.1 Cigarette smoke extract (CSE) treatment upregulates PAFr expression in bronchial epithelial cells

Epithelial cells treated with 1% CSE exhibited significantly higher PAFr expression per cell compared to untreated cells as measured by immunofluorescence ($p < 0.0001$) (Figure 6.1). PAFr mRNA expression (relative to three housekeeping genes) was 2.93 ± 0.18 ($n = 4$) in BEAS-2B cells exposed to CSE, and 2.28 ± 0.33 ($n=4$) in control cells (Figure 6.1).

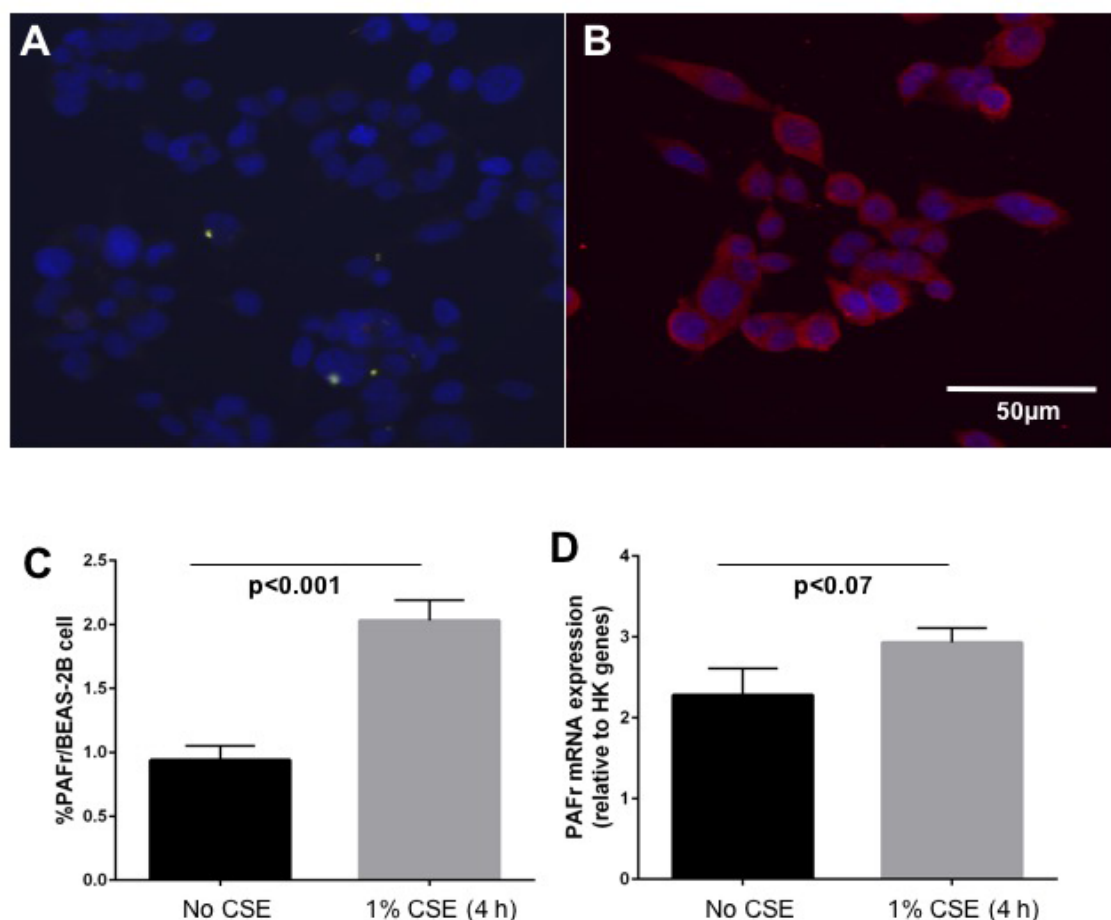


Figure 6.1 Cigarette smoke extract increases PAFr-expression in BEAS-2B bronchial epithelial cells.

(A) Untreated BEAS-2B cells. (B) Microscopy of BEAS-2B cells exposed to CSE (1%, 4 hours). All micrographs show BEAS-2B cells with PAFr expression (anti-PAFr monoclonal antibody; 1:100, red) and nuclei stained with DAPI (1:5000, blue). Magnification = 400x. (C) Percentage area of PAFr

compared to cell nuclei area was measured using area of interest cell-tracing calculations. (D) PAFr mRNA expression relative to housekeeping genes. Bars represent standard error of the mean.

6.3.2 CSE significantly increases NTHi and *S. pneumoniae* adherence to BEAS-2B cells

Exposure of BEAS-2B cells to 1% CSE for 4 hours significantly enhanced epithelial cell binding of *S. pneumoniae* and of both NTHi strains (Figure 6.2). Regression analyses were performed on levels of epithelial PAFr expression against bacterial adhesion. This demonstrated that there was a linear relationship between an increase in PAFr protein levels and elevated levels of adhesion to CSE-pretreated lung cells for all three bacterial strains ($r \geq 0.9$; $p < 0.0001$) (Figure 6.3).

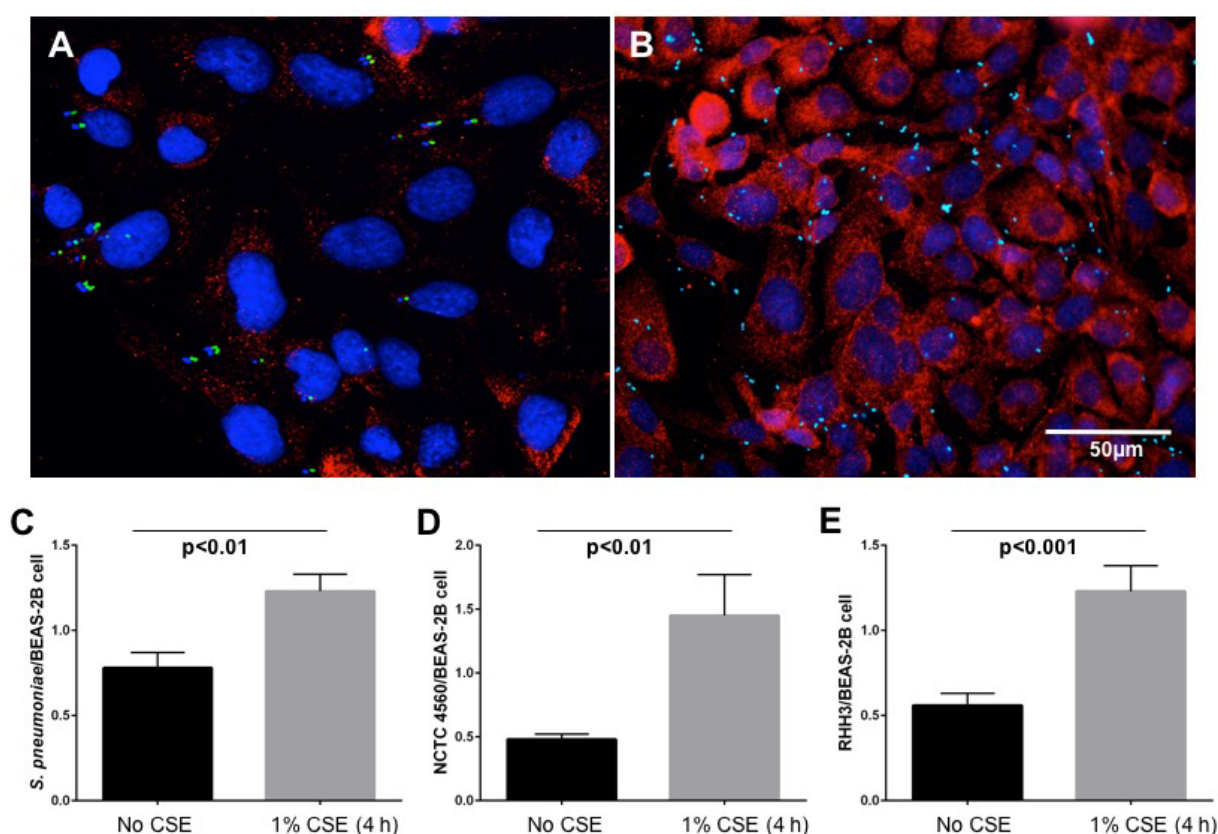


Figure 6.2 Cigarette smoke extract increases the adhesion of NTHi strains and *S. pneumoniae* to BEAS-2B cells (n=4).

(A) Control BEAS-2B cells showing few FITC-tagged *S. pneumoniae* 132 cells adhering to BEAS-2B cells. (B) BEAS-2B cells exposed to CSE (1%) show increased attachment of FITC-tagged *S. pneumoniae* to BEAS-2B cells. All

micrographs show BEAS-2B cells with PAFr expression (anti-PAFr monoclonal antibody; 1:100, red) and nuclei stained with DAPI (1:5000, blue). Magnification=400x. (C) *S. pneumoniae* clinical isolate 132. (D) NTHi reference strain NCTC-4560. (E) NTHi clinical isolate RHH3. Bars represent standard error of the mean.

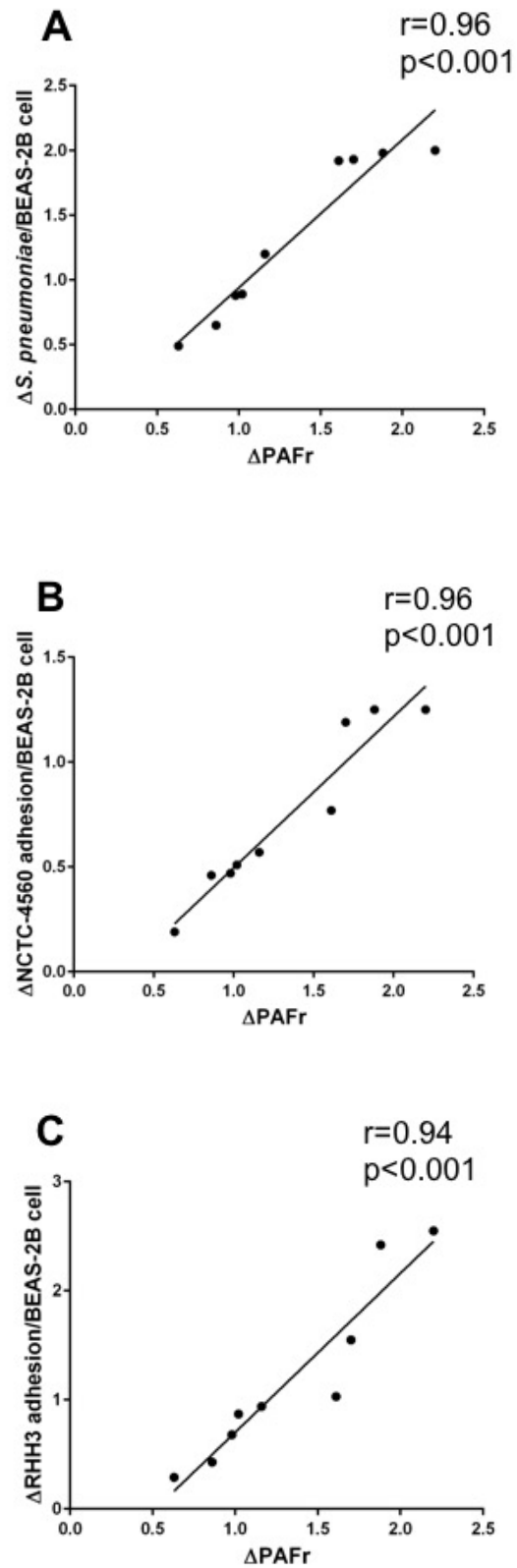
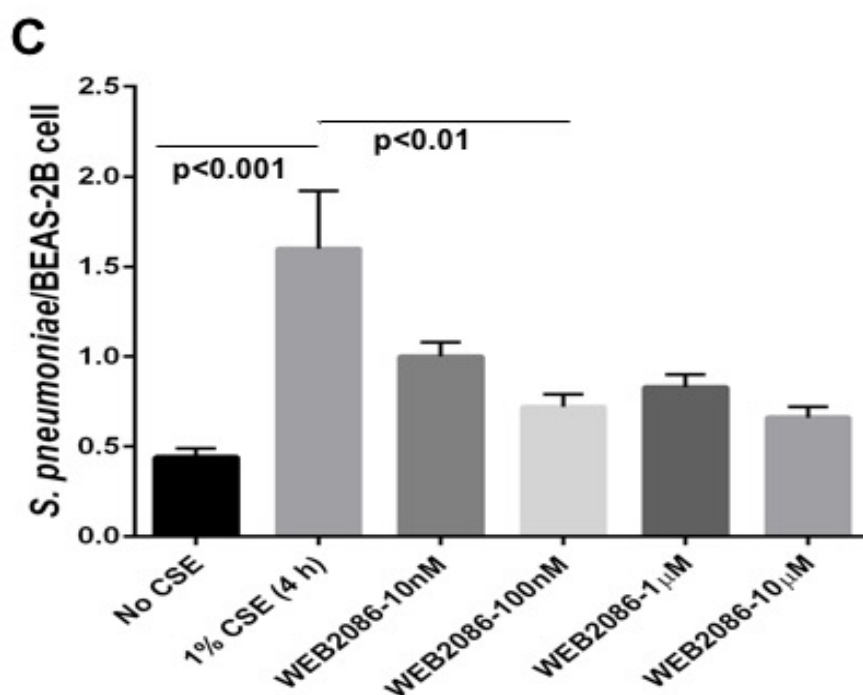
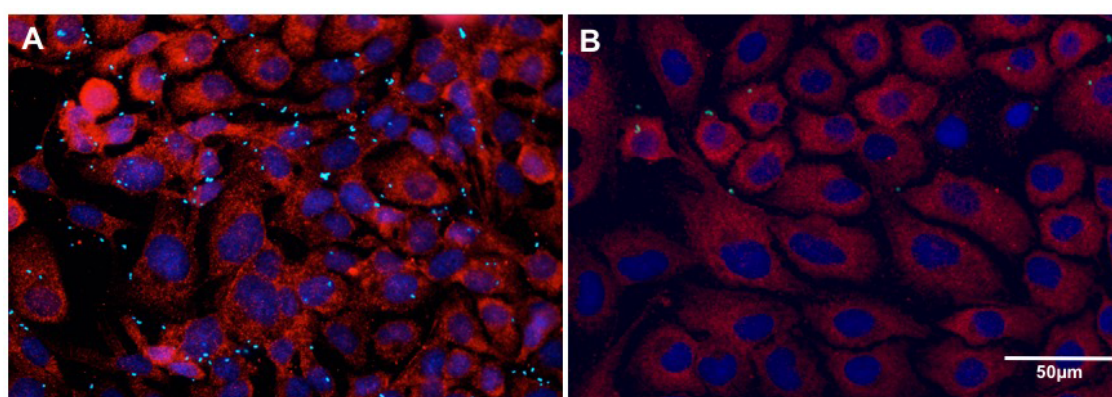


Figure 6.3 Correlations between change in PAFr protein expression and change in the number of adherent bacteria to BEAS-2B cells.

6.3.3 WEB-2086 reduces the adhesion of *S. pneumoniae* and NTHi to CSE-treated bronchial epithelial cells

WEB-2086 decreased *S. pneumoniae* adhesion to CSE-treated BEAS-2B cells back to control levels at a concentration of 100 nM ($p=0.0058$) (Figure 6.4). WEB-2086 at 10 μM , but not lower concentrations, also reduced adhesion of the reference strain of NTHi to CSE-treated cells to control levels ($p<0.0001$) (Figure 6.4). Adhesion of the clinical strain of NTHi to CSE-treated BEAS-2B cells was reduced to control levels by WEB-2086 at a concentration of 1 μM . WEB-2086 was non-toxic to epithelial cells even at the highest concentration tested *i.e.* $\text{IC}_{50}>10\text{ }\mu\text{M}$.



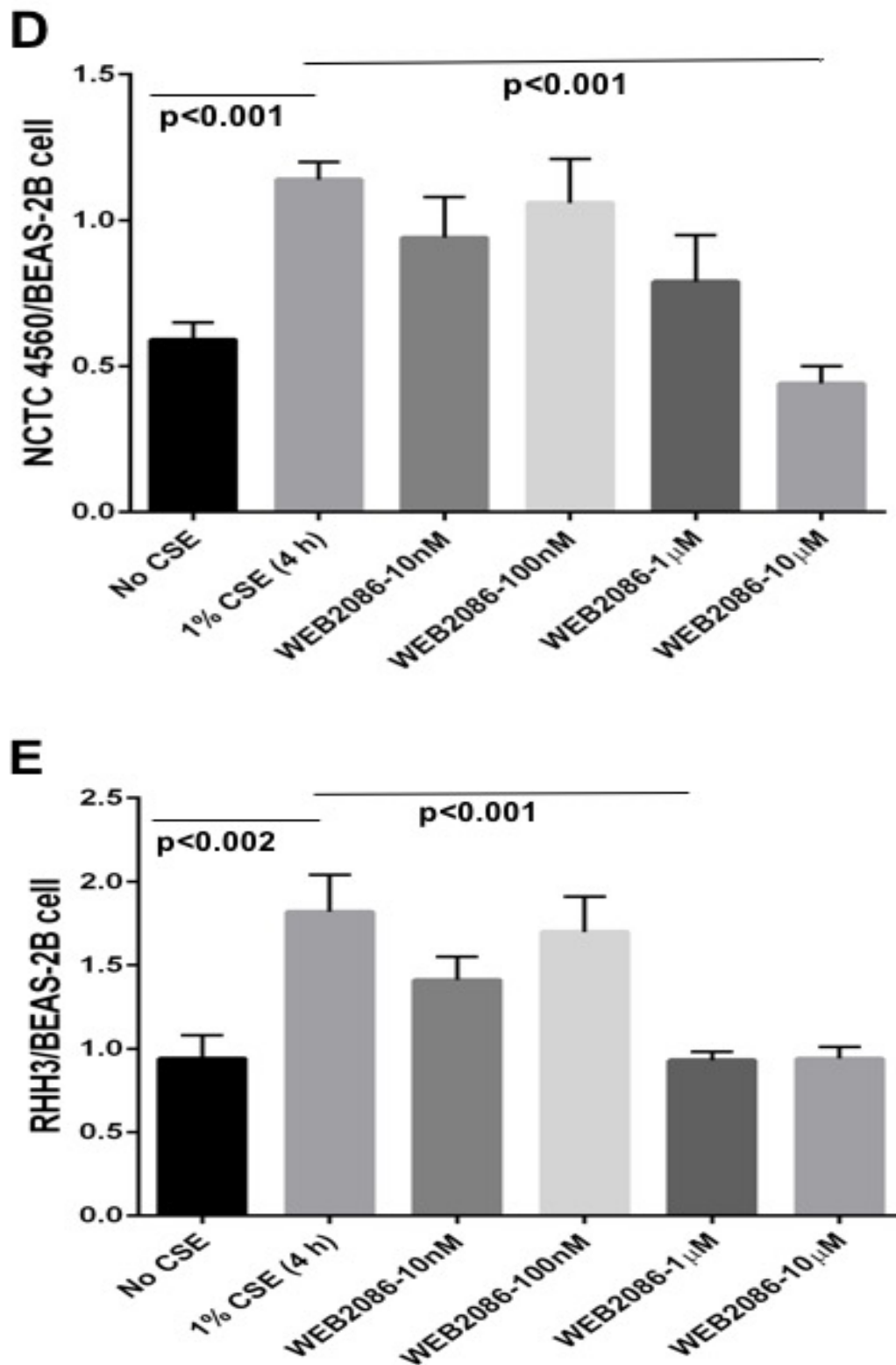


Figure 6.4 Inhibitory effect of WEB-2086 on the adhesion of bacterial strains to CSE-treated BEAS-2B cells.

(A) BEAS-2B cells exposed to CSE (1%) showing attachment of FITC-tagged *S. pneumoniae* clinical isolate 132. (B) Reduced attachment of *S. pneumoniae* bacterial cells to CSE-treated BEAS-2B cells due to application of PAFr antagonist, WEB-2086. (C) Significant reductions in *S. pneumoniae* adhesion to CSE-treated cells were observed in the presence of 100 nM, 1 μ M and 10 μ M WEB-2086. (D) A significant reduction in NTHi reference strain (NCTC-4560) adherence to CSE-treated was observed in the presence of 10 μ M WEB-2086. (E) Significant reductions in NTHi clinical isolate (RHH3) adherence to CSE-treated cells were observed in the presence of 1 μ M and 10 μ M WEB-2086. Bars represent standard error of the mean.

6.3.4 Docking WEB-2086 to a molecular model of PAFr

Initial models of PAFr produced by MODELLER and I-TASSER were very similar (r.m.s. deviation of 3 Å over 295 C α atom pairs), and predicted that the same set of amino acids would contribute to the ligand binding site. A final consensus model of PAFr was generated in MODELLER using NTR1 and CCR5 as template structures (Figure 6.5A), taking advantage of regions of local high sequence/structure similarity to each template. The natural PAF ligand and WEB-2086 were docked to the model using the AUTODOCK Vina package. In a search of the whole molecular surface, both PAF and WEB-2086 consistently docked into the same deep cavity on the predicted extracellular face of the PAFr model (Figure 6.5A). The cavity is substantially larger than the WEB-2086 compound (Figure 6.5B) and a fine-grained docking search of this pocket identified a number of potential docking poses.

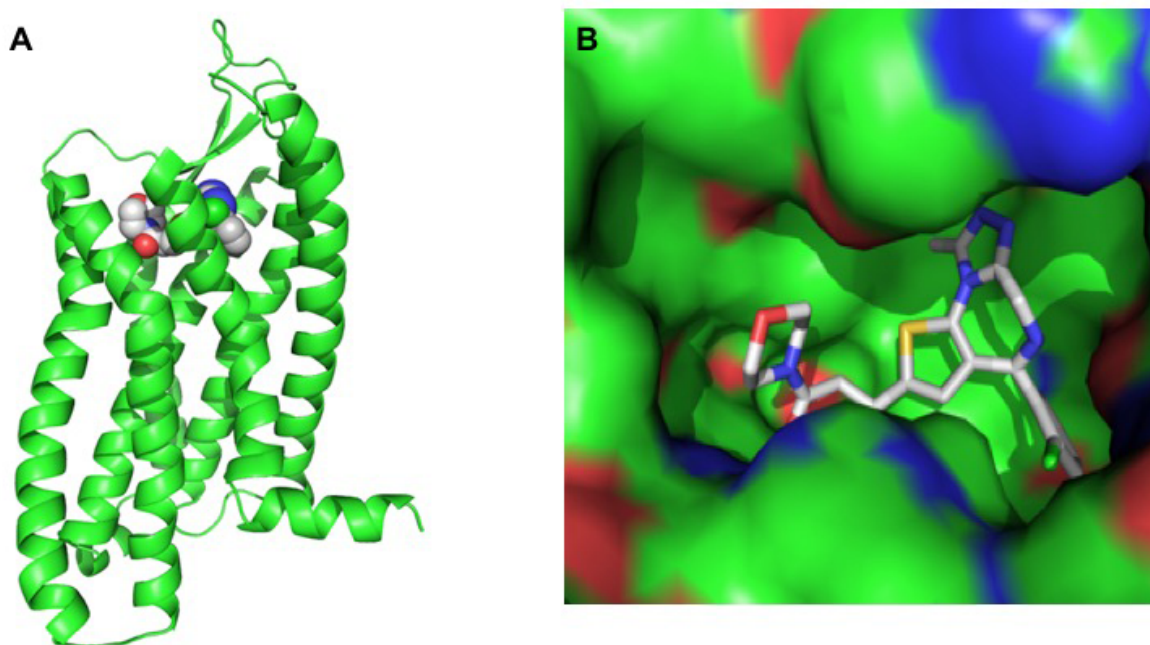


Figure 6.5 *In silico* analyses of PAFr structure and ligand binding.

(A) Ribbon representation of the molecular model of the PAFr receptor (residues 8–309), based on the template structures of NTR1 (PDB 4buo) and CCR5 (PDB 4mbs). The expected extracellular face is towards the top of the page. The docking pose for WEB-2086 with the highest predicted binding affinity is located in a deep cleft in the central axis of the receptor. (B) Surface representation of the central ligand binding cleft in the PAFr model, shown from above. WEB-2086 is shown in stick representation.

6.4 Discussion

In the present study, I found that *in vitro* exposure to cigarette-smoke extract increased PAFr expression and adhesion of NTHi and *S. pneumoniae* to bronchial epithelial cells. Regression analysis identified a correlation between PAFr expression and levels of bacterial adherence. As PAFr expression increased on the surface of the epithelial cells, the number of NTHi and *S. pneumoniae* cells binding per BEAS-2B cell increased linearly.

Addition of PAFr antagonist (WEB-2086) reduced the adherence of both respiratory bacterial pathogens down to control levels, but large range of

effective doses depending on bacterial strains. Interestingly, the adherence of the *S. pneumoniae* clinical isolate was inhibited at a lower concentration of PAF antagonist compared to the NTHi strains tested. There were no consistent differences in the slope of the relationship between PAFr receptor upregulation and bacterial attachment, for the different strains tested, to suggest an inter-species difference in PAFr-dependent bacterial adhesion. A larger number of isolates of both species would need to be investigated to ascertain any species- or strain-specific susceptibility to WEB-2086, but it should be noted that varying levels of adhesion to airway epithelial cells have been observed across different clinical isolates of NTHi (Marti-Llitas et al. 2011).

This is the first study reporting the efficacy of WEB-2086 in specifically reducing NTHi adherence to CSE pre-treated lung cells. This is also true for pneumococci although a similar inhibitory effect was observed for WEB-2086 in an epithelial cell culture model of pneumococcal infection in which PAFr was upregulated by particulate air pollution (Mushtaq et al. 2011). The available data suggest that exposure to cigarette smoke may promote infection of the lung epithelium of COPD patients by the main bacterial drivers of AECOPD, NTHi and *S. pneumoniae*, via upregulated PAFr, though changes in ChoP expression may also play a part. I have also discussed the variation in ChoP expression that may lead to altered NTHi adherence and invasion, and reduced resistance to pulmonary clearance (chapter 2, page 44-55).

In this work, I tested a well-evaluated PAFr antagonist for its ability to inhibit the binding of NTHi and *S. pneumoniae* to CSE pre-treated airway epithelial cells. WEB-2086 was effective in significantly reducing the adhesion of both respiratory pathogens to CSE-exposed bronchial epithelial cells with demonstrated PAFr upregulation. Interestingly, the bacterial adhesion seen in the presence of WEB-2086 was comparable to control levels observed for the cultured epithelial cells for which PAFr expression had not been induced through CSE exposure. In terms of drug tolerability, PAF antagonists, including WEB-2086, have been shown to be safe in both animals and

humans, even at high doses over several months (Cellai et al. 2006; Cellai et al. 2002; Kato, M et al. 2004). Similarly, I did not find WEB-2086 to be toxic to BEAS-2B cells even at the highest effective level.

There are a few limitations to this study. I used a commercial airway epithelial cell line, and further work is now needed on the anti-infective effects of WEB2086 using primary human cells, especially from smokers. Secondly, the ligand-docking studies are reliant on an *in silico* model of PAFr which was derived from the tertiary structure of related GPCRs. X-ray crystallographic work is required to confirm the specific PAFr-WEB2086 interactions.

In summary, I investigated the anti-adhesion activity of WEB-2086 towards NTHi and *S. pneumoniae*, two major respiratory pathogens on airway epithelium primed by CSE to upregulate PAFr expression. I demonstrated strong association between PAFr upregulation and bacterial adhesion; the latter of which was reduced to control levels in the presence of the PAFr antagonist WEB-2086. Understanding the events leading to the colonisation of the lower respiratory tract of COPD patients with NTHi and pneumococcus, and to subsequent increased local and systemic inflammation, may lead to translation into new management strategies for COPD. Given the apparent tolerance of WEB-2086 in human subjects and its ability to block adhesion of *S. pneumoniae* and NTHi to respiratory epithelial cells, I am now keen to see clinical exploratory proof-of-concept studies of PAF antagonists undertaken in healthy individuals, and then in COPD patients to ascertain if they positively affect day-to-day symptoms as well as prevent or ameliorate AECOPD. PAFr-antagonists represent a potential non-antibiotic, anti-infective drug for therapeutic interventions in COPD patients, especially perhaps those who are prone to frequent AECOPD, and indeed in smokers more generally who are vulnerable to episodes of pneumonia.

Chapter 7

The main rhinovirus respiratory tract adhesion site (ICAM-1) is upregulated in smokers and patients with chronic airflow limitation (CAL)

7.1 Introduction

COPD is seriously complicated by viral as well as bacterial infections. Viruses are detected in 40 to 60% of COPD exacerbations (AECOPD) in PCR-based studies (Sykes, Mallia & Johnston 2007), and are associated with more severe exacerbations in terms of symptoms, resulting in longer recovery times and greater likelihood of hospitalization. Human rhinoviruses (HRVs) make up approximately 50% of all viruses isolated from COPD patients (Greenberg, SB et al. 2000). Furthermore, HRV prevalence and load at COPD exacerbations are significantly higher than in the stable state (George et al. 2014). In COPD patients experimentally infected with HRV, the sputum viral load correlates with sputum neutrophilia and interleukin-8 levels (Mallia, P. et al. 2011), i.e., the activation of the innate immune system characteristic of these AECOPD episodes.

Respiratory tract epithelium is the primary target for viral pathogens and ICAM-1 has been reported as the major surface receptor for HRVs in ~60% serotypes, essential for host-cell entry, while low-density-lipoprotein receptor and related molecules serve as an entry receptor for only 10% of HRV serotypes (Ledford et al. 2004). Very recently, a novel receptor (CDHR3) has been suggested to be relevant to HRV-C attachment (Bochkov et al. 2015), although its relevance to HRV infection has not been fully established.

ICAM-1 is a member of the immunoglobulin (Ig) superfamily that contains five Ig-like domains, a transmembrane domain, and a short cytoplasmic tail (Springer 1990). I have extensively discussed the characteristics of ICAM-1 in chapter 2 (page 57-63). The importance of ICAM-1 in physiologically normal smokers has been reported previously (chapter 2, page 57-63). Moreover, anti-ICAM-1 antibody has been shown to inhibit major (group A and B) HRV

replication *in vitro*, as well as HRV-induced inflammation and lung virus RNA levels in mice model (Traub et al. 2013), confirming a crucial potential role for ICAM-1 as a viral facilitator. However, the expression of ICAM-1 has not been directly investigated in large or small airways in COPD patients, which could be crucial for understanding the susceptibility to and effect of viral infections in the natural history of COPD.

Evidence indicates that ICAM-1 also serves as an adhesion molecule for *Haemophilus influenzae* (via bacterial P5 fimbriae), which is the main bacterial pathogen in COPD, again especially in AECOPD (Sethi & Murphy 2008). Thus, ICAM-1 is an attractive target to block not only virus-receptor binding, but also contribute to perhaps checking ICAM-1-mediated NTHi adhesion to respiratory cells. This is of particular interest as there is some existing work in the field, but looking at only healthy smokers rather than COPD.

In the present study, I have taken a somewhat new direction for human work, and tested the potential for clinical relevance of some of these previous observations. I have investigated whether ICAM-1 expression is upregulated in the epithelium of both the large and small airways, and also in the alveoli, in both “normal” smokers and in patients with airflow obstruction, including individuals with established COPD.

7.2 Overview of materials and methods

I employed classical immunohistochemical methods to evaluate PAFr expression in the large airway epithelium in bronchial biopsies collected from well phenotyped participants (as detailed in methodology section, chapter 3).

7.3 Results

7.3.1 ICAM-1 expression in epithelium of large and small airways

Compared to normal controls, epithelial staining was increased in the apical areas (large airways: $p < 0.006$; small airways: $p < 0.004$) in NLFS, whereas in the CAL group, heavy ICAM-1 expression was observed throughout the airway epithelium (large airway: $p < 0.001$; small airway: $p < 0.001$), including

both the apical and basal cells, though basal cell staining was heaviest (Figure 7.1 & Figure 7.2). ICAM-1 expressing cells were significantly increased in the epithelium of CAL group, both in the large ($p<0.007$) and small airways ($p<0.02$), when compared to NLFS group. For all current smoker groups (NLFS and CAL analysed separately), there were positive relationships between pack-year smoking history and ICAM-1 expression, for both the large and small airways ($r=0.50$; $p<0.03$) (Figure 7.3).

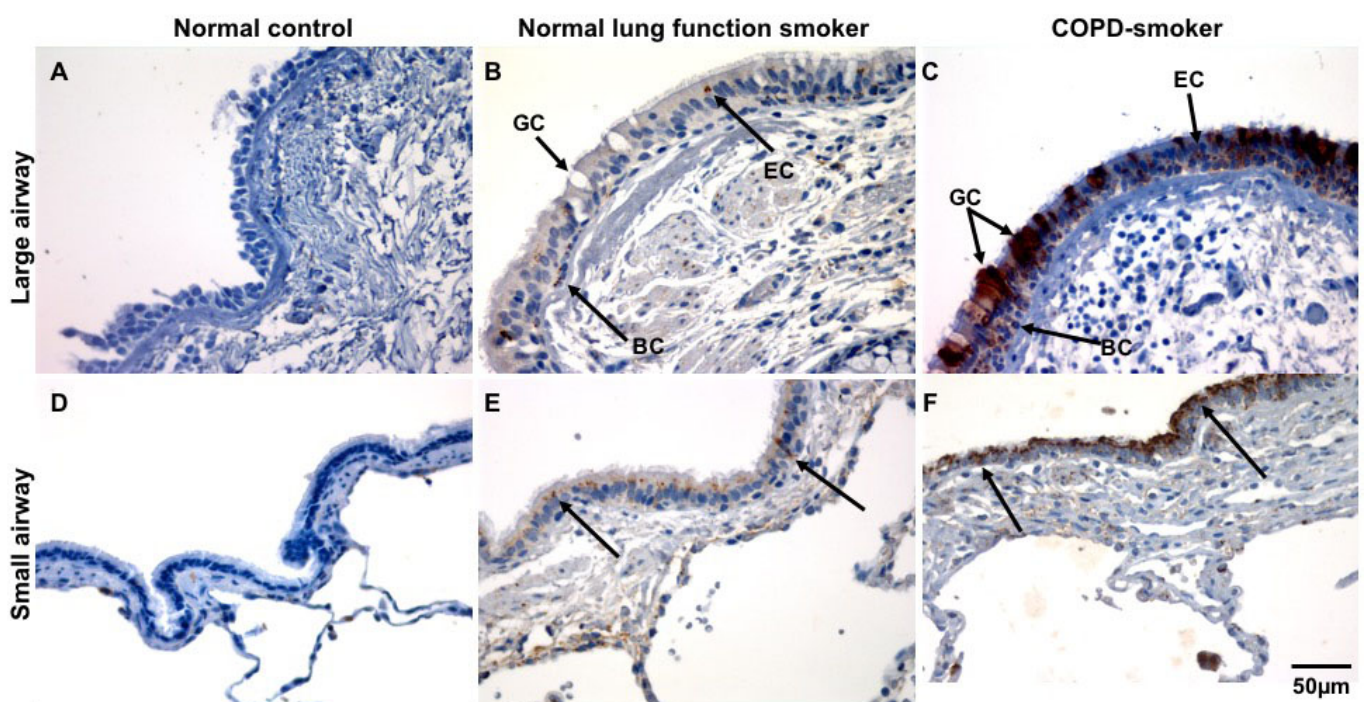
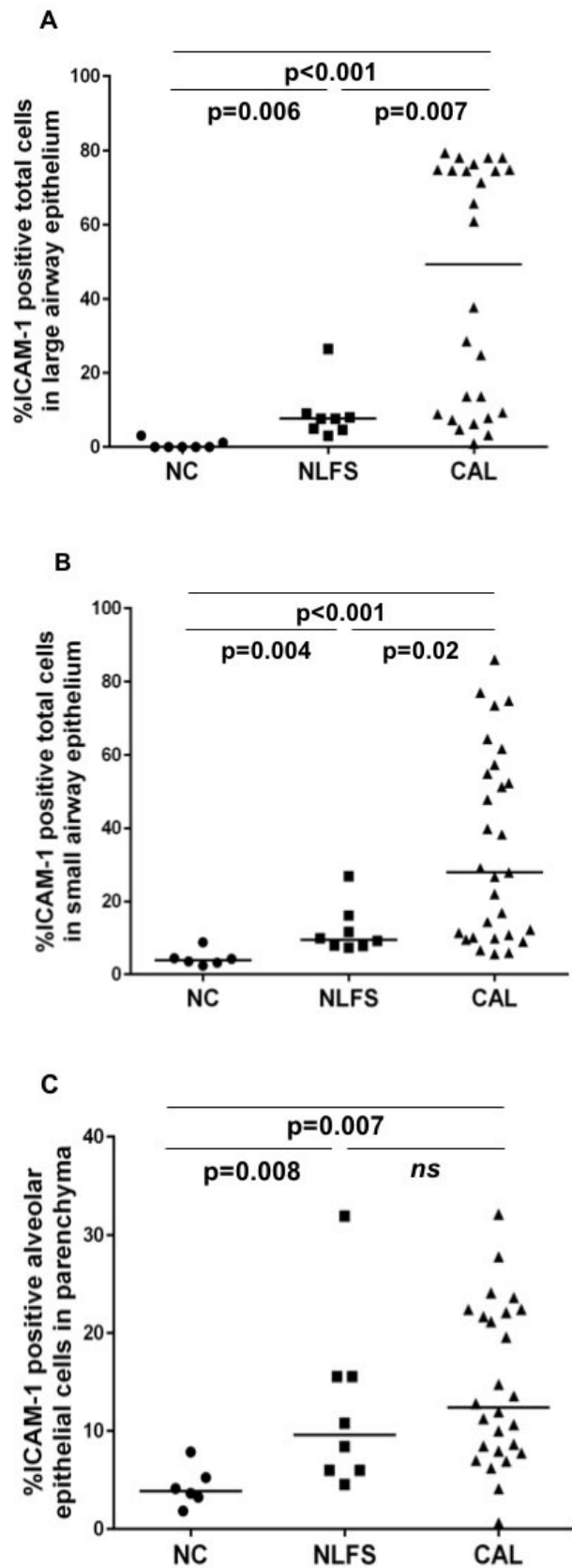


Figure 7.1 Intercellular adhesion molecule-1 (ICAM-1) expression in epithelium of large and small airways.

(A, D) Representative section of small airway from a never smoker showing negligible ICAM-1 staining. (B,E) Typical normal lung function smoker showing positive staining (showed by black arrow). (C,F) Typical COPD-smoker showing extensive ICAM-1 staining (black arrow). Magnification= $\times 400$. BC: basal cells; EC: epithelial cells; GC; goblet cells.

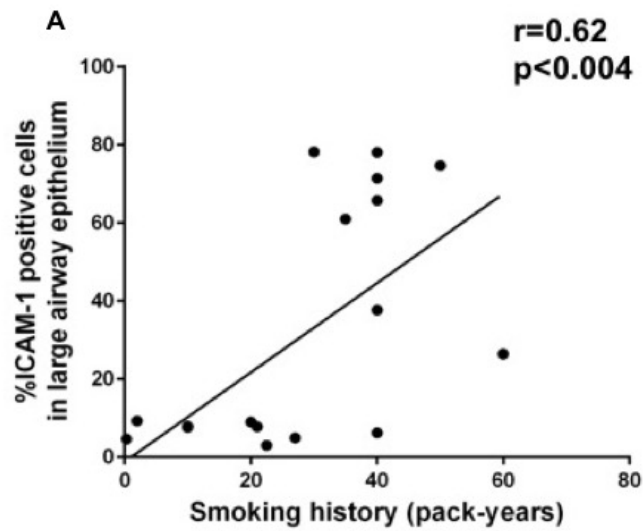


(A) Large airway epithelium

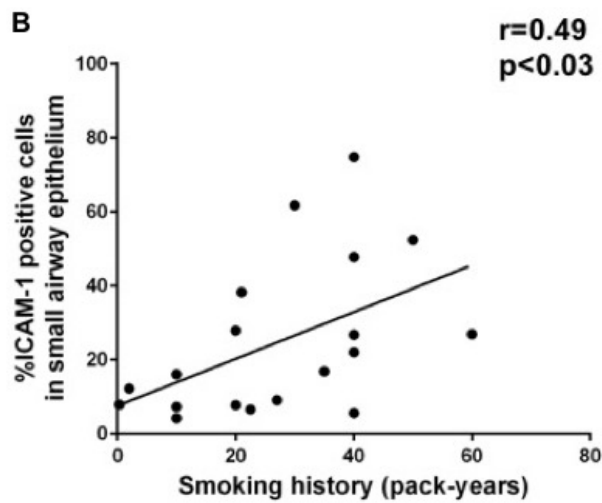
(B) Small airway epithelium

(C) Lung alveolar epithelial cells

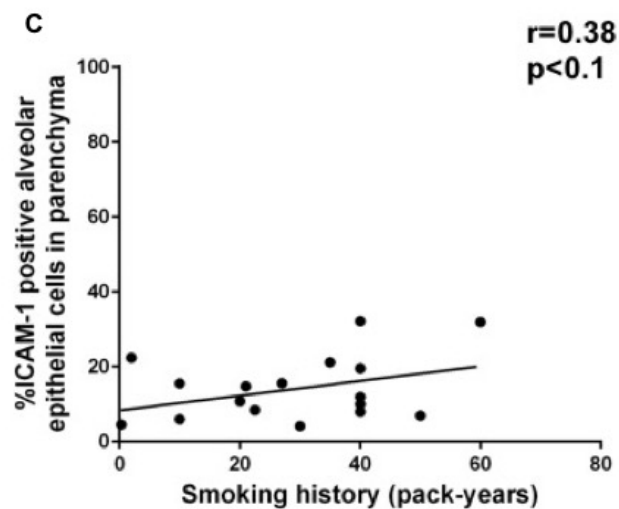
Figure 7.2 Quantification of ICAM-1-expressinf cells in cross-sectional study.



(A) Large airway epithelium



(B) Small airway epithelium



(C) Lung alveolar epithelial cells

Figure 7.3 Correlations between total epithelial cells positive for ICAM-1 with pack-years of smoking history.

7.3.2 ICAM-1 positive cells in the airway Rbm

The Rbm in smokers and especially in COPD has been reported as hypercellular (Sohal, Sukhwinder S. et al. 2010), and this was true in this study also. However, the only significant increase in the number of ICAM-1 expressing cells in the Rbm was observed in small airways of the CAL group ($p<0.02$). These ICAM-1 positive cells were especially related to cells with a morphological fibroblast-like phenotype Figure 7.4, though the number of individuals with high ICAM-1 expression in large airways did seem greater in NLFS and especially in CAL in large airways.

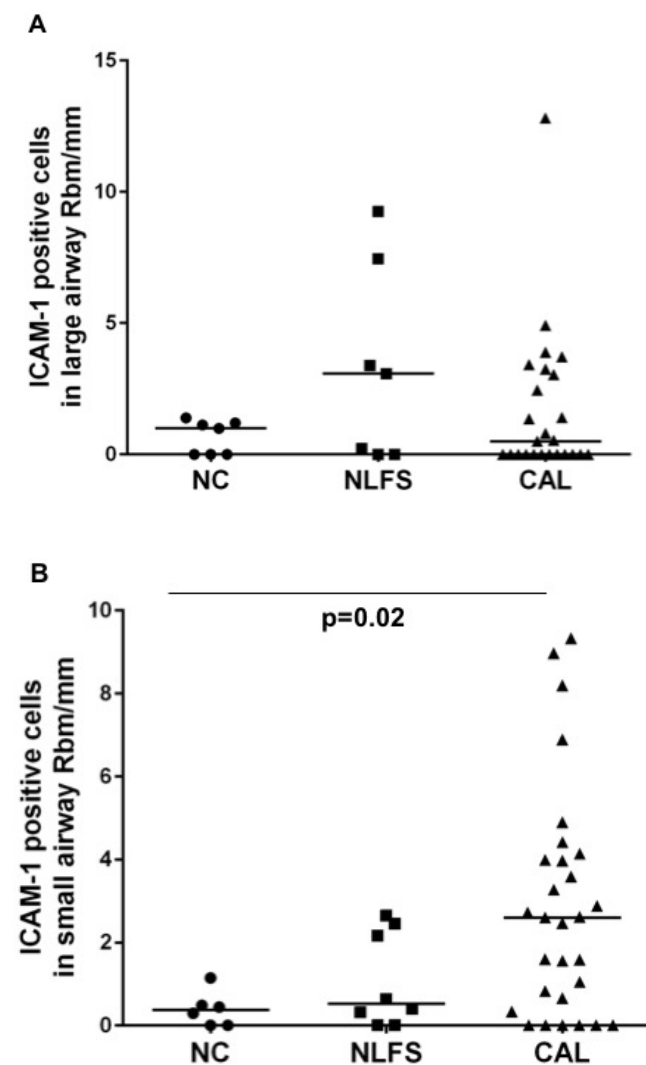


Figure 7.4 ICAM-1 expressing cells in Rbm.

(A) Large airway. (B) Small airway.

7.3.3 ICAM-1 expression in the goblet cells and sub mucosal glands in the large airways

A novel finding that we did not expect, was that goblet cells in the large airways of NLFS ($p<0.05$) and CAL ($p<0.004$) patients showed intense ICAM-1 expression compared to control tissues (Figure 1 & Figure 4). In addition, the staining intensity was significantly higher for the CAL group ($p<0.04$), compared with NLFS and NC groups combined. Similarly, we observed increased ICAM-1 positivity in the submucosal glands in tissues from the CAL group compared with both controls sets ($p<0.05$), and there was also higher staining intensity ($p<0.03$) (Figure 5).

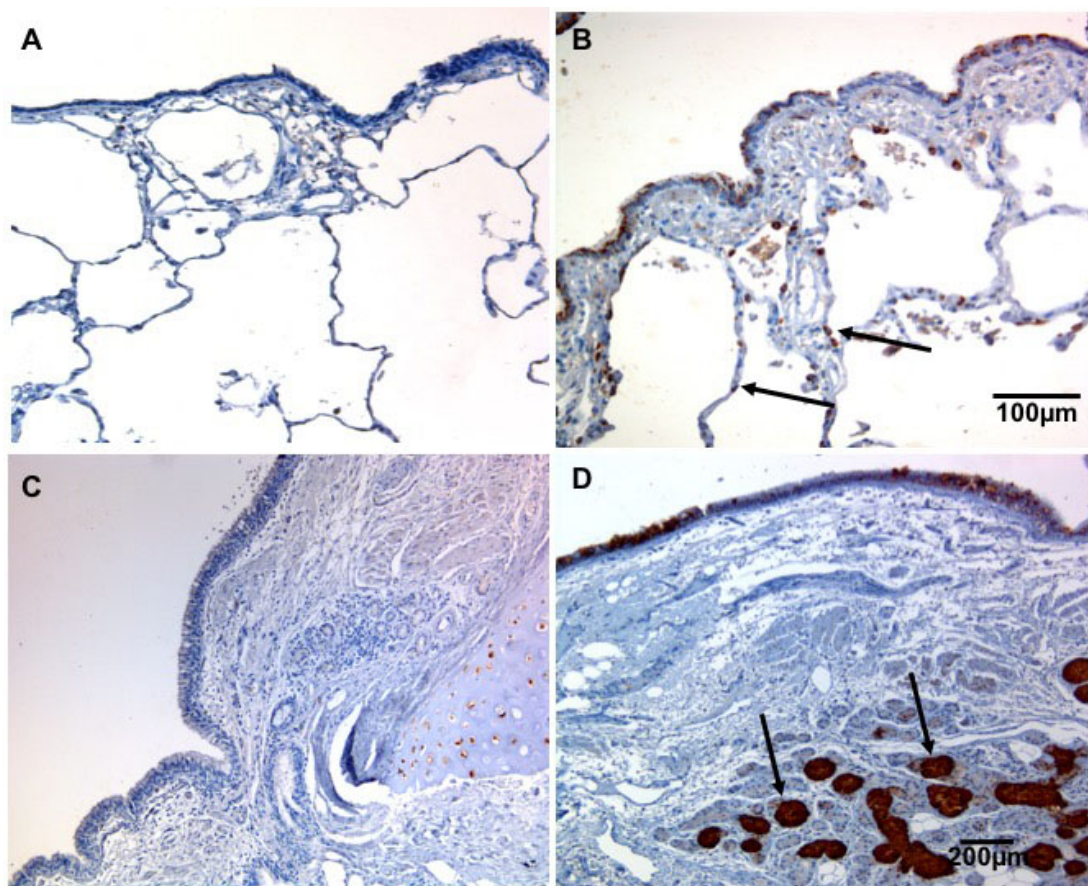


Figure 7.5 Photomicrograph showing ICAM-1 expression.

(A-B) lung alveolar epithelium. (C-D) submucosal glands. (A) Representative sections from a never smoker. (B) Typical COPD-smoker showing positive staining in alveolar epithelial cells (mainly type II). (C) normal lung function smoker. (D) COPD-smoker showing extensive ICAM-1 staining in submucosal glands. Magnification: A-B= $\times 100$; C-D= $\times 200$.

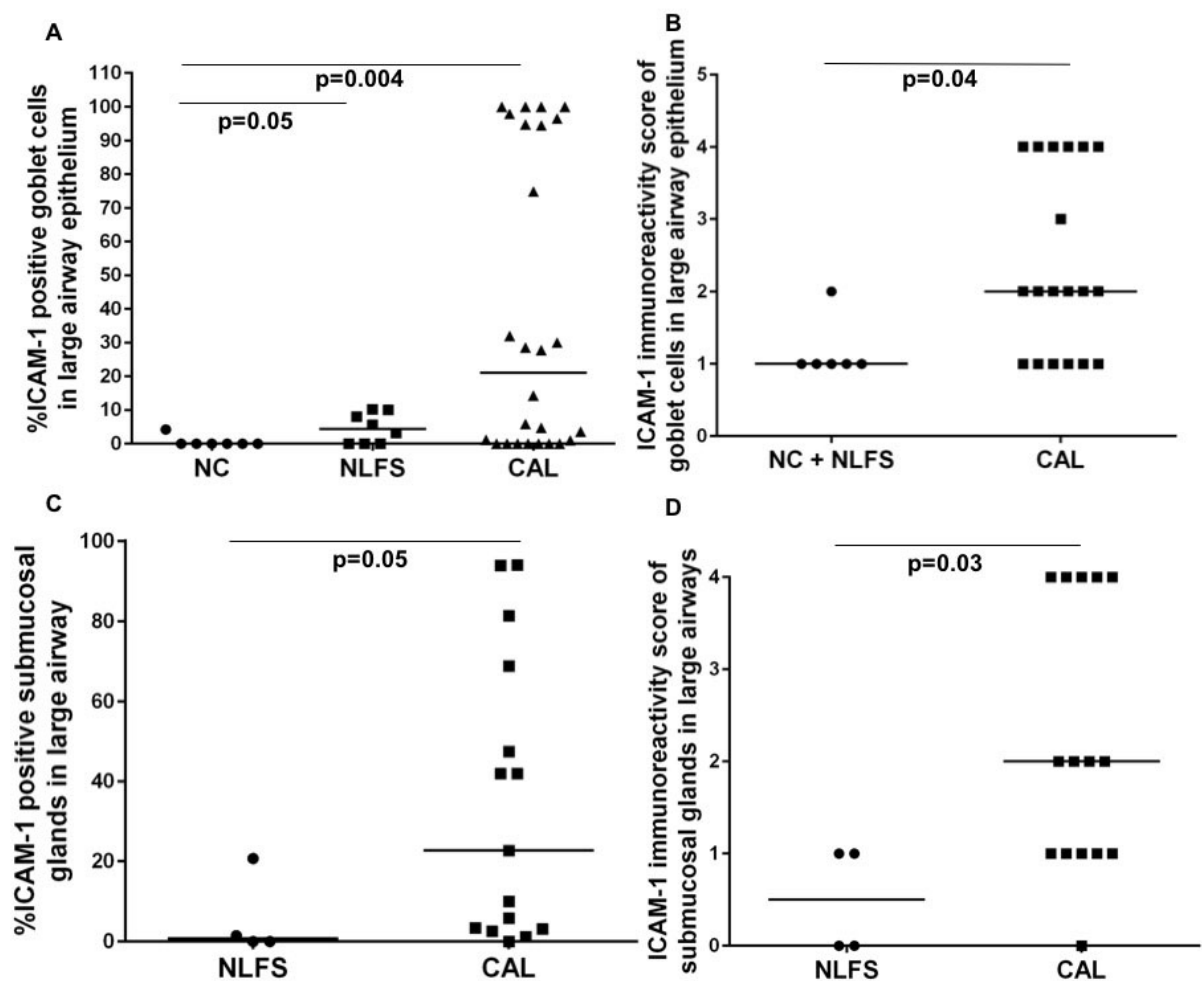


Figure 7.6 Quantification of ICAM-1 expression in goblet cells and submucosal glands in the cross-sectional study.

(A) ICAM-1 expressing goblet cells in large airway epithelium. (B) Intensity of ICAM-1 staining of goblet cells in large airway epithelium. Only ICAM-1 positive goblet cells are included in this comparison. (C) ICAM-1 expressing submucosal glands. (D) Intensity of ICAM-1 staining of submucosal glands.

7.3.4 ICAM-1 expression in the alveolar cells in lung parenchymal

Overall, approximately 15-30% of total epithelial cells, most frequently but not exclusive type II cells, were found to be positive in both the NLFS ($p < 0.008$) and CAL ($p < 0.007$) groups, compared with normal tissue; on an average, approximately 4% cells were positively stained for ICAM-1 in the control group (Figure 2 & 4).

7.3.5 Cigarette smoke extract (CSE) treatment upregulates ICAM-1 expression in bronchial epithelial cells

ICAM-1 mRNA expression (relative to three housekeeping genes) was increased in BEAS-2B cells exposed to CSE ($p<0.03$; $n=4$) compared to control cells (Figure 6). Moreover, CSE exposure significantly increased ICAM-1 protein expression per cell compared to untreated cells ($p<0.003$) (Figure 6).

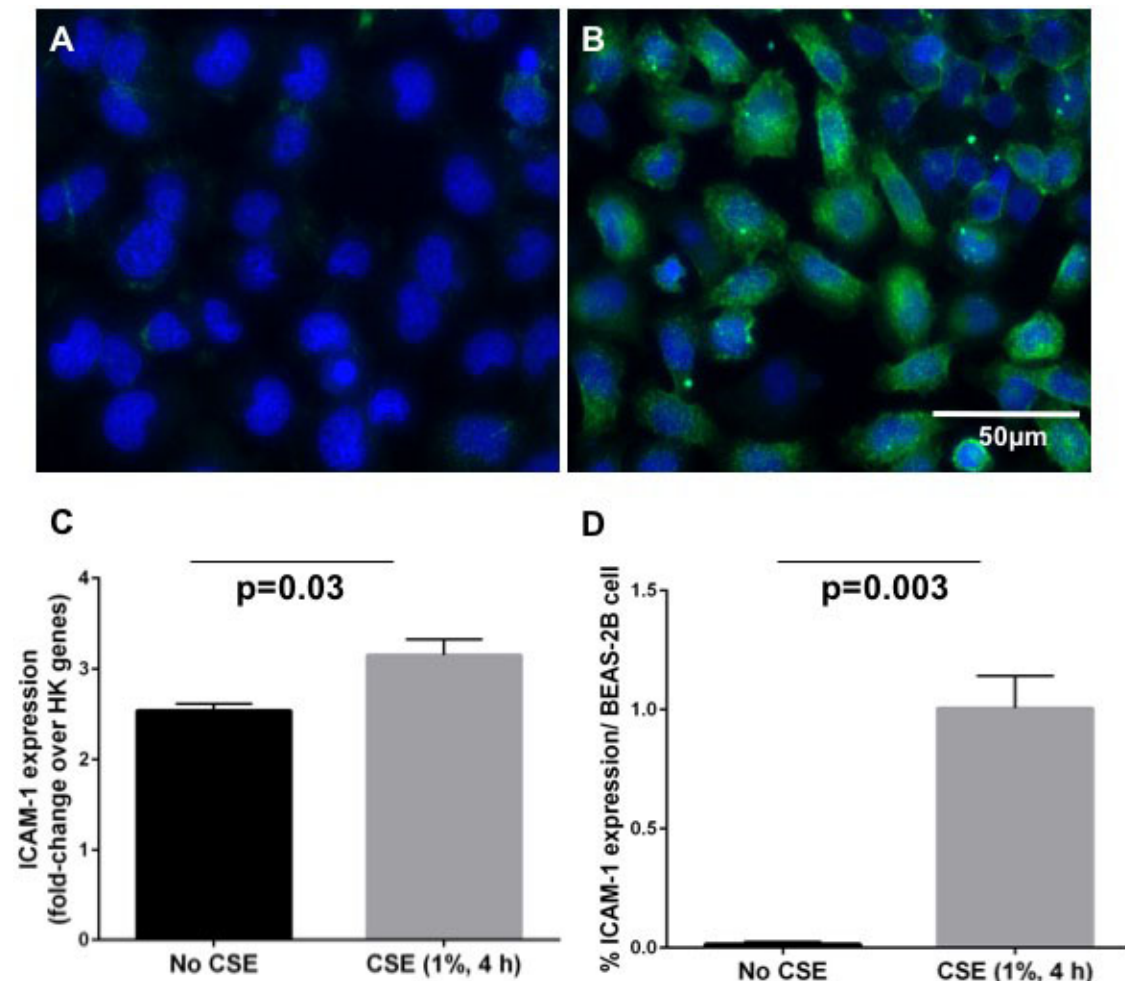


Figure 7.7 Photomicrograph showing ICAM-1 expression in bronchial epithelial cells.

(A) BEAS-2B control cells. (B) BEAS-2B cells pretreated with CSE (1%, 4 hours). (C) CSE 1% increases ICAM-1 transcript level, assessed by quantitative RT-PCR and normalised for three housekeeping gene. (D) CSE (1%) also increases protein expression on cultured epithelial cell culture, quantified by computerized image analysis software. Magnification=400x. (Blue: nuclear stain DAPI; Green: Alexa Fluor 488 showing ICAM-1 stain).

7.4 Discussion

This is the first comprehensive report of increased ICAM-1 protein expression in epithelium of both the large and small airways in smokers but especially in patients with chronic airflow limitation (CAL), including individuals with definite COPD. There is some up-regulation in the alveolar epithelium of the lung parenchyma but this is less marked than in the airways, and with no difference between normal smokers and obstructed smokers or ex-smokers. I also found increased ICAM-1 expression in goblet cells in large airway from smokers and CAL patients. Furthermore, ICAM-1 expression in the submucosal glands was increased in CAL group compared to smokers.

I analysed all individuals with airflow obstruction together as a single group, as there was no obvious difference in regard to ICAM-1 expression in the respiratory tract between those with SAD only and those with more advanced and classic COPD. Moreover, ICAM-1 expression, both at the mRNA and protein level, was upregulated in cultured bronchial epithelial cells exposed to cigarette smoke extract. These findings, taken as a whole may be crucial for understanding the vulnerability of smokers and especially patients with airflow obstruction to airway infections, specifically with HRV and NTHi, although for the latter, platelet-activating factor receptor (PAFr) upregulation may be of even greater importance (chapter 4 and chapter 5). Clinical relevance of increased ICAM-1 expression in the pathogenesis of smoking-related airway diseases including COPD has been suggested previously, but mainly through indirect evidence, which is summarized on pages 57-63. Briefly, HRV has been detected in lower airway specimens such as sputum from children with wheezy bronchitis (Horn, Reed & Taylor 1979), and brushed cells from allergic volunteers experimentally infected with RV16 (Gern et al. 1997) by RT-PCR and culture. Moreover, compared with normal control, cultured airway epithelial cells from patients with COPD showed increased susceptibility to RV infection, and also higher levels of mRNAs encoding ICAM-1 (Schneider et al. 2010). Mechanistically, HRV itself upregulated membrane-bound ICAM-1 expression via NF- κ B-dependent mechanisms, in normal primary human bronchial epithelial cell cultures (Papi & Johnston 1999).

In this study, I did not find any correlation between cellular ICAM-1-expression in the airway and either age or lung function in CAL group, but ICAM-1 expression in both the large and small airways was significantly correlated with smoking history, with a wide range of pack-years represented.

My data also suggest that in addition to respiratory epithelium, large airway goblet cells and submucosal glandular cells may also be involved in HRV pathogenesis. Empirically, airway viral infection results in mucus hypersecretion, which may play a role in the pathogenesis of severe airway obstruction in AECOPD. Notably, I showed increased ICAM-1 expression in goblet cells and submucosal glands in the large airway of smokers, but especially so in CAL patients. Furthermore, the intensity of staining of these cells was markedly heavier in the CAL group. Previous research has shown that HRV infection could upregulate ICAM-1-mRNA and inflammatory cytokines in submucosal gland cells, and, an anti-ICAM-1 antibody blocked both RV infection as well as post-infection production of these cytokines in submucosal gland cells (Yamaya et al. 1999). Thus, airway goblet and submucosal glands may be an important potential target of RV infection, and given that there is marked hypertrophy and hyperplasia of this glandular tissue in COPD, it again adds to the vulnerability of these patients to HRV at acute exacerbations.

In addition to being a receptor for the majority of HRV serotypes, ICAM-1 may also serve as an adhesion receptor for *Haemophilus influenzae* (Avadhanula, Rodriguez, Devincenzo, et al. 2006). Blocking cell surface ICAM-1 with specific antibody significantly reduced the adhesion of NTHi to A549 respiratory epithelial cells (Avadhanula, Rodriguez, Ulett, et al. 2006). It has been shown that NTHi itself upregulates ICAM-1 expression as incubation of respiratory epithelial cells with NTHi increased ICAM-1 expression by four-fold, both at the mRNA and surface protein levels (Frick et al. 2000). Pretreatment of bronchial epithelial cells (BEAS-2B) and primary bronchial epithelial cells in culture with NTHi significantly up-regulated ICAM-1 and enhanced adhesion of HRV to these cells (Gulraiz et al. 2015). These studies in relation to NTHi did not take into account the possibility of co-regulation of

ICAM-1 with Platelet Activating Factor receptor (PAFr), which we have previously suggested to be the main airway adhesion site for pathogenic *Haemophilus* with a high correlation (chapter 6, page 120). Work on potential interactions between these two adhesion systems is now urgently needed, which could lead to novel non-antibiotic, anti-infective therapeutic strategies that could break some of these postulated vicious cycles involving microbial adhesion molecules.

In this study, I have found that alveolar epithelial cell ICAM-1 expression was increased equivalently in smokers and the CAL group, with type II cells being the predominant cell type affected. Empirically, staining was much less marked than in the airways. Burns et al also previously reported increased ICAM-1 expression in type II pneumocytes in mice lung tissue exposed to *S. pneumoniae* (Burns, A R, Takei & Doerschuk 1994), potentially facilitating CD18-dependent neutrophil emigration into sites of acute inflammation. They did not explore the possibility of increased microbial vulnerability, which is likely to exist, though the major potential for infection would seem to be airways, which fits with clinical experience.

The strengths of the present study include the use of abundant and relevant human tissue in well phenotyped individuals with mild-to-moderate obstructive airway disease, focusing on pathogenic mechanisms in relatively early disease with few confounding factors such as chronic bacterial infection or emphysema. I had robust numbers to give sufficient power to detect these findings, and this was confirmed by the strong statistical outcomes.

There are also a few limitations. Firstly, the study was cross-sectional at a single time point and longitudinal studies would be better able to track the variability of ICAM-1 expression within individuals and if this relates to viral infectivity and the natural history of smoking-related airway disease and especially AECOPD prospectively. Secondly, our control subjects were somewhat younger on average than the clinical groups, but ages over-lapped substantially between groups and there was no suggestion of a relationship between ICAM-1 expression and age within any group. Finally, there was no microbiological component; we need to investigate variability in viral

adherence to respiratory epithelial cells in relation to ICAM-1 expression.

In conclusion, epithelial ICAM-1 expression is upregulated throughout the respiratory tract in smokers, but is especially marked in subjects with chronic airflow obstruction, even when mild, and then in both the large and small airways. ICAM-1 expression in goblet cells and submucosal glands is also markedly increased in smokers and patients with CAL. There is an increase but less marked than in the airways in ICAM-1 expression in alveolar epithelium, especially in Type-2 cells, but this is only a smoking effect. Increased expression of ICAM-1 in the respiratory tract, and mostly so in the airways, could be a crucial risk factor for infection in the respiratory tract with this common viral agent in smokers and especially in AECOPD. Whether this increase in ICAM-1 expression encourages NTHi adherence to human airways and lung tissue needs further assessment. Translational research in this area is still in its infancy but has huge potential to provide new therapeutic targets to modify clinical management of smoking-related airflow limitation. Thus, further clinical research on novel anti-ICAM-1 therapies is now warranted.

Chapter 8

Summary and Conclusions

8.1 Overview of results

Respiratory tract infections are acknowledged to be one of the important contributing factors in episodes of acute exacerbations in COPD (AECOPD). There is also growing evidence that chronic bacterial colonization also plays a key role in the progression of COPD. My thesis reports novel findings from investigations of microbial receptors in lung tissues, especially airway epithelium, collected from smokers and COPD patients. I propose that increased expression of highly strategic adhesion sites for microbes are major pathological changes contributing to increased susceptibility of these individuals to chronic airway colonization and persistence of pathogens in the host. These changes in the airways were not described as comprehensively in the literature before, and I believe this thesis will significantly improve the current understanding of airway infections in COPD, and potentially lead to new therapeutic approaches.

My thesis is divided into seven main sections. First two chapters outline the current evidence on the role of microbial infections in COPD. Third chapter details the methodological approach employed, and also contains the demographics of study participants. There are four main chapters elaborating my study results. In the first two results section (chapter 4 and chapter 5), I investigated the PAFr-protein expression in the respiratory tract epithelium (large and small airways plus lung parenchyma) of smokers and COPD patients, which was compared to physiologically healthy smokers and healthy non-smokers. I also quantified PAFr-expressing inflammatory cells in the parenchymal tissue. These *ex-vivo* findings using human tissues led me to investigate the effects of cigarette smoke extract in an *in-vitro* epithelial cell culture model (using commercially available BEAS-2B cells). In chapter 6, I confirmed that CSE increased PAFr mRNA transcripts as well as protein in these cells. This increase in PAFr significantly correlated with bacterial (for both NTHi and *S. pneumoniae*) adhesion to CSE-pretreated epithelial cells.

Furthermore, blocking PAFr expressed on CSE-treated epithelial cells reduced the absolute number of bacterial cells adhering to BEAS-2B cells. Finally in the last data chapter (chapter 7), I demonstrated an increase in the major adhesion receptor (ICAM-1) for ~60% HRV serotypes, in the epithelium of large and small airways, as well as lung parenchyma in smokers and patients with chronic airflow limitation (CAL), the majority with frank COPD. Again, I confirmed these findings in smoking-induced epithelial cell culture model (at both mRNA level and protein expression pattern). The summary of each chapter is briefly discussed below.

8.1.1 PAFr-expression in large airways

This was the first study to comprehensively report PAFr-expression in large airway epithelium in COPD patients with full appropriate controls. This study was cross-sectional and I studied the bronchial biopsies from well phenotyped study groups, which was a major strength of the study (chapter 4). I demonstrated that PAFr expression was significantly increased in smokers, but especially so in subjects with COPD. Moreover, the increase in PAFr-protein expression correlated with smoking history, and further, smoking cessation in COPD does not reduce the PAFr-expression levels back to normal. In this chapter, I was also able to utilize tissue material from a previous randomized control trial to assess the effect of ICS (1000mcg/day Fluticasone/placebo for 6 months) on PAFr-expression. I found that median PAFr-expression was increased after six months of ICS therapy in COPD patients, although statistically non-significant. This observation could be quite important and should encourage future research, given the fact that risk of pneumonia is increased in COPD patients on ICS therapy for reasons not really understood. The risk of lower airway bacterial infections in smokers and COPD patients could potentially be due to increased expression of PAFr, at least in some individuals.

8.1.2 PAFr-expression in small airways

This was also the first study to report on PAFr-expression in the epithelium of both the small airway and alveoli in COPD patients (chapter 5). A total of 45 individuals participated in this study. Epithelial PAFr-protein expression was increased in small airways of smokers, but especially marked in patients with COPD. I also evaluated the PAFr-expression in lung parenchyma (both type I and type II alveolar epithelial cells), and found that PAFr-expressing alveolar epithelial cells were increased in smokers, compared to normal controls. However, this was not further enhanced in COPD patients. I also observed some PAFr-expressing inflammatory cells in the lung parenchyma, but these were not increased in COPD patients. Again, pack-years of smoking were strongly correlated with small airway epithelial PAFr-expression in all current smokers. Thus, both current smoking and a history of smoking seem important for PAFr expression.

8.1.3 Effect of a PAFr antagonist in reducing the attachment of respiratory bacterial pathogens

I developed an *in vitro* epithelial cell culture model to confirm whether cigarette smoke extract affects PAFr-expression, and subsequent whether bacterial adhesion to cells (chapter 6). I found that treatment of bronchial epithelial cells (BEAS-2B) with CSE increased PAFr-expression, which was indeed significantly associated with increased bacterial (both NTHi and *S. pneumoniae*) adhesion. The levels of PAFr-protein expression significantly correlated with the number of bacteria adhering to epithelial cells pre-treated with CSE. Moreover, a specific PAFr-antagonist, WEB-2086 reduced the epithelial adhesion by both these bacteria to levels observed for non-CSE exposed cells. Moreover, WEB-2086 was found to be non-toxic towards the bronchial epithelial cells. In collaboration with a protein biochemist, I was able to carry out *in silico* analyses of PAFr protein, which allowed identification of the binding pocket for PAFr/WEB-2086 in the predicted PAFr tertiary structure. WEB-2086 represents an innovative class of candidate drugs for inhibiting

PAFr-dependent lung infections by the main bacterial drivers of smoking-related COPD.

This was the first study to report competitive inhibition of WEB-2086 towards NTHi adhesion to CSE-treated BEAS-2B cells. WEB-2086 represents an innovative class of candidate drug for inhibiting PAFr-dependent lung infections by the main bacterial drivers of smoking-related COPD (Figure 8.1).

Proof of concept clinical studies are needed, but first we need more data from well phenotyped clinical groups but using human airway primary cultures. It is also important to know whether currently used medications affect PAFr upregulation; how most of them have benefits long term in COPD is rather enigmatic.

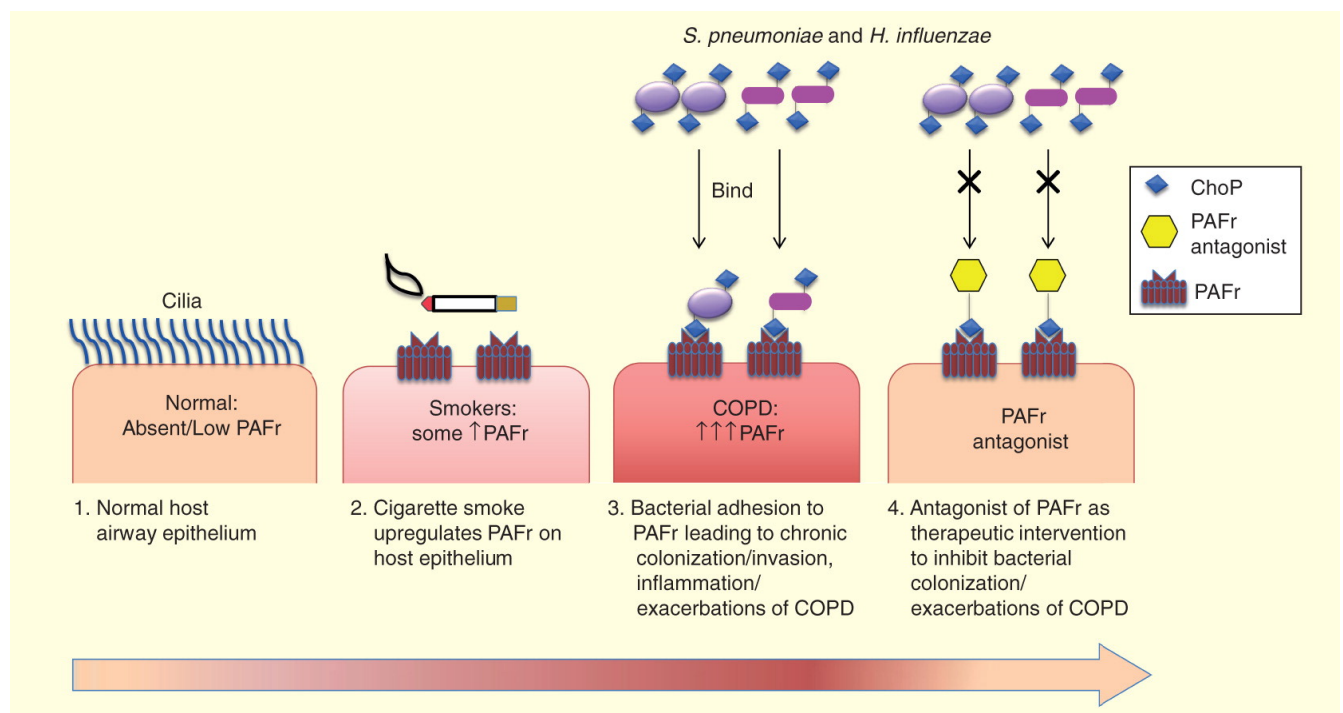


Figure 8. 1 Platelet activating factor receptor (PAFr) as a potential therapeutic target.

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8.1.4 ICAM-1 expression

The final analysis involved the assessment of epithelial ICAM-1 expression in both the large and small airways, as well as lung parenchyma, of smokers and patients with chronic airflow limitation (CAL) (chapter 7). The CAL group included both frank COPD (smokers and ex-smokers) and subjects with small airway narrowing only. I found that ICAM-1 expression was upregulated throughout the respiratory tract (both large and small airway epithelium) in smokers, but was especially marked in subjects with chronic airflow obstruction, even when mild including CAL. Notably, I also showed increased ICAM-1 expression in goblet cells and submucosal glands in the large airway of smokers, but especially so in CAL patients. Furthermore, the intensity of staining of these cells was markedly heavier in the CAL group.

Assessment of ICAM-1 in lung parenchyma was also undertaken. There was an increase, but less markedly so than in the airways, in ICAM-1 expression in alveolar epithelium, especially in Type-II alveolar epithelial cells, but this was only a smoking effect, i.e. no extra expression in CAL/COPD. Similar to PAFr expression, epithelial ICAM-1 expression in both large and small airway, was significantly correlated with pack-years of smoking history in both normal epithelium, 'healthy' smokers and CAL subjects.

Finally, ICAM-1 expression, both at the mRNA and protein level, was upregulated in cultured bronchial epithelial cells (BEAS-2B) exposed to cigarette smoke extract.

These findings taken on a whole may be crucial for understanding the vulnerability of smokers and especially CAL patients to airway viral infections, specifically with major group HRV (group A and B), but also potentially with NTHi.

8.2 Concluding remarks

In my thesis, I hypothesized that airway epithelial microbial receptors (namely PAFr and ICAM-1) would be upregulated in physiologically 'normal' smokers and especially in COPD patients. I showed that this is the case, and further validated my *ex-vivo* lung tissue data with *in-vitro* epithelial cell culture

studies. I have provided detailed quantitative information on both the major microbial receptors in smokers and COPD and their relation to smoking history. The expression patterns for both PAFr and ICAM-1 seemed to be highly variable within each study groups, but it was significantly different between phenotypic groups. Moreover, quitting smoking does not seem to completely normalise the adhesin-profile, especially in COPD subjects.

Thus, “classic” respiratory pathogens (both bacteria and viruses) seem to have evolved extremely important strategies to survive within their respective niches, by specifically adhering to attachment proteins such as PAFr and ICAM-1. The initial pathogen binding to the airway epithelial surface may aid in chronic colonization/acute infection of the host.

My project also presented experimental evidence that it is possible to block PAFr in epithelial cell cultures in a way that reduces adherence of bacterial pathogens to these cells. This opens the possibility that these receptors could be targeted therapeutically in COPD, and even in Pneumococcal pneumonia. Translational research in this area is still in its infancy but has huge potential to provide new therapeutic targets.

The knowledge gained during my PhD studies at University of Tasmania will enable me to develop strategies to further understand the microbial pathogenesis in COPD. I now plan to collect primary lung epithelial cells from human volunteers (normal controls, normal smokers and COPD patients); it would be useful to assess the PAFr expression pattern as well as investigate the effects of COPD medication (LABA, LAMA and ICS combinations) on these cells. Furthermore, infection assays will be carried out to further investigate the efficacy of PAFr-antagonist (WEB-2086) and novel compounds that colleagues in Hobart have designed. The investigation of molecular mechanisms involved in the adhesion of bacterial pathogen to host cell will be investigated in mouse models. Another major research aspect that needs to be investigated is the variability of both the amount and localization of ChoP on the cell wall of clinical isolates of NTHi in the same cohort of patients over time, and how these factors correlate with the natural history of COPD. ChoP gene (Lic A-D) mRNA expression in NTHi clinical isolates, longitudinally

obtained from COPD patients over one/two years, and would enable me to assess this bacteria-associated ChoP variability in the pathogen. Moreover, collection of clinical data would allow correlation of variation in ChoP expression with disease trajectories. Furthermore, additional studies on PAFr-expressing macrophages, which I reported in chapter 5, could be really valuable, as this particular aspect of the host immune system has not been investigated to date, and it could be interesting to see whether PAFr-expression on macrophages aids in clearance of bacterial pathogens or impairs bacterial clearance from the host.

Although the investigation of novel anti-PAFr/ICAM-1 therapies are challenging, this is now essential in order to better manage both chronic infection in COPD airways as well as AECOPD while avoiding the real risk of increasing antibiotic-resistance in major bacterial pathogens, such as *S. pneumoniae* and non-typeable *H. influenzae*. I intend that all these potential studies, leading from my PhD outcomes, will project my career forwards, but to do this effectively I will need to cultivate an interest in this work among clinical colleagues that I work with in the future.

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Appendix

A1. ICAM-1 expression in large and small airway epithelium.

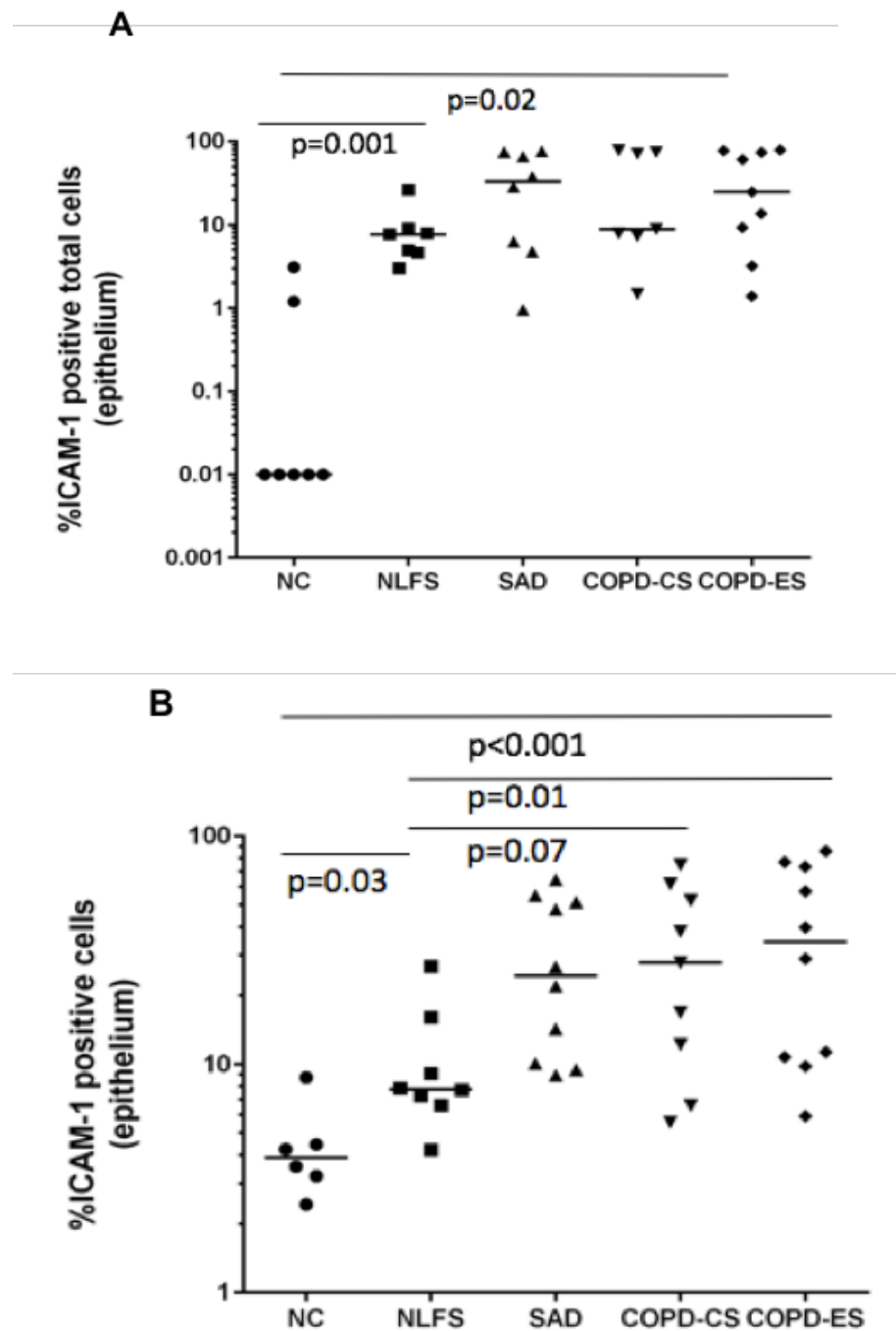


Figure A.1. Epithelial ICAM-1 expressing cells **(A)** large airways; **(B)** small airways.

NC: normal control; NLFS: normal lung function smoker; SAD: small airway disease; COPD-CS: COPD-current smoker; COPD-ES: COPD ex-smoker